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(71) Applicant: RIBOZYME PHARMACEUTICALS, INC. [US/US]; 2950 Wilderness Place, Boulder, CO 80301 (US).																																																																						
(72) Inventors: DRAPER, Kenneth, G.; 4619 Cloud Court, Boulder, CO 80301 (US). CHOWRIRA, Bharat; 3250 O'Neal Circle, B-25, Boulder, CO 80301 (US). McSWIGGEN, James; 4866 Franklin Drive, Boulder, CO 80301 (US). STINCHCOMB, Daniel, T.; 7203 Old Post Road, Boulder, CO 80301 (US). THOMPSON, James, D.; 2925 Glenwood Drive #301, Boulder, CO 80301 (US).																																																																						
(74) Agents: WARBURG, Richard, J. et al.; Lyon & Lyon, First Interstate World Center, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).																																																																						
(54) Title: METHOD AND REAGENT FOR INHIBITING HUMAN IMMUNODEFICIENCY VIRUS REPLICATION																																																																						
<table border="1"> <thead> <tr> <th>RPI</th> <th>3'-end</th> <th>CORE</th> <th>STEM/LOOP II</th> <th>5'-end</th> <th>ACTIVITY</th> </tr> </thead> <tbody> <tr> <td>1197</td> <td>.</td> <td>.</td> <td>.</td> <td>.</td> <td>1</td> </tr> <tr> <td>1200</td> <td>OMe</td> <td>.</td> <td>.</td> <td>OMe</td> <td>0.7</td> </tr> <tr> <td>1315</td> <td>DNA</td> <td>.</td> <td>DNA</td> <td>DNA</td> <td>0.02</td> </tr> <tr> <td>1370</td> <td>Allyl</td> <td>.</td> <td>.</td> <td>Allyl</td> <td>1</td> </tr> <tr> <td>1371</td> <td>Aro</td> <td>.</td> <td>.</td> <td>Aro</td> <td>0</td> </tr> <tr> <td>1414</td> <td>OMe/FU</td> <td>FU7</td> <td>.</td> <td>OMe/FU</td> <td>0.5</td> </tr> <tr> <td>1368</td> <td>.</td> <td>TT</td> <td>.</td> <td>.</td> <td>0.7</td> </tr> <tr> <td>1431</td> <td>OMe</td> <td>NH2-U4-T</td> <td>.</td> <td>OMe</td> <td>0.5</td> </tr> <tr> <td>1394</td> <td>OMe</td> <td>.</td> <td>.</td> <td>OMe</td> <td>0 (46-mer)</td> </tr> <tr> <td>1285</td> <td>.</td> <td>.</td> <td>-4 bp</td> <td>.</td> <td>0.1 (28-mer)</td> </tr> </tbody> </table>					RPI	3'-end	CORE	STEM/LOOP II	5'-end	ACTIVITY	1197	.	.	.	.	1	1200	OMe	.	.	OMe	0.7	1315	DNA	.	DNA	DNA	0.02	1370	Allyl	.	.	Allyl	1	1371	Aro	.	.	Aro	0	1414	OMe/FU	FU7	.	OMe/FU	0.5	1368	.	TT	.	.	0.7	1431	OMe	NH2-U4-T	.	OMe	0.5	1394	OMe	.	.	OMe	0 (46-mer)	1285	.	.	-4 bp	.	0.1 (28-mer)
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(57) Abstract																																																																						
An enzymatic nucleic acid molecule which cleaves an immunodeficiency virus RNA in a gene required for viral replication, e.g., the <i>nef</i> or <i>tat</i> gene regions.																																																																						

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DESCRIPTIONMethod and Reagent for Inhibiting  
Human Immunodeficiency Virus ReplicationBackground of the Invention

This application is a continuation-in-part of Draper, U.S. Serial No. 07/882,886, filed May 14, 1992, and U.S. Serial No. 08/103,423, filed August 6, 1993, both entitled  
5 "Method and Reagent for Inhibiting Human Immunodeficiency Virus Replication," assigned to the same assignee as the present application, the whole of which (including drawings) are hereby incorporated by reference herein and are abandoned in favor of the present application.

10 This invention relates to the use of ribozymes as inhibitors of human immunodeficiency virus (HIV) replication, and in particular, the inhibition of HIV-1 replication. See e.g., Draper et al., PCT/WO93/23569 hereby incorporated by reference.

15 Acquired immunodeficiency syndrome (AIDS) is thought to be caused by infection with the virus HIV-1. At present, it is treated by administration of the drug azidothymidine (AZT), which is thought to slow the progress of, but not cure, the disease. AZT resistant  
20 strains of HIV-1 are found to develop after a year of treatment. In some patients AZT has limited efficacy and may be found intolerable. More recently, drugs such as dideoxyinosine (DDI) and dideoxycytidine (DDC) have been tested as treatments for AIDS. None of these compounds  
25 reduce the viral load in patients, but they do treat the disease symptoms.

The following is a discussion of relevant art, none of which is admitted to be prior art to the pending claims. Rossi et al., 8 Aids Research and Human  
30 Retroviruses 183, 1992, provide a review of the use of ribozymes as anti-HIV-1 therapeutic agents. They state:

An emerging strategy in the treatment of viral infections is the use of antisense DNA or RNA to pair with, and block expression of viral transcripts. RNA, in addition to being an informational molecule, can also possess enzymatic activity. Thus, by combining anti-sense and enzymatic functions into a single transcript, it is now possible to design catalytic RNAs, or ribozymes, which can specifically pair with virtually any viral RNA, and cleave the phosphodiester backbone at a specified location, thereby functionally inactivating the viral RNA. In carrying out this cleavage, the ribozyme is not itself altered, and is thus capable of recycling and cleaving other molecules, making it a true enzyme. There are several different catalytic motifs which possess enzymatic activity, and each one of these can be incorporated into an enzymatic antisense with site-specific cleavage capabilities.

Rossi et al. also state that studies have demonstrated that a hammerhead ribozyme targeted to the gag gene RNA in the vicinity of the translational initiation codon is capable of specifically cleaving that target in a complex milieu of total cellular RNA. With reference to identification of ribozyme targets in HIV-1 they state that mRNAs for the two regulatory proteins tat and rev are clearly targets of choice, and that they are examining potential ribozyme cleavage sites in the tat mRNA, as well as in the exon shared by tat and rev. In addition, they state:

A rational approach to the problem of target selection involves the following criteria. First, one should select a functionally important target, such as tat, rev, int, psi (packaging site), or the tRNA<sup>lys</sup> priming site. Once a gene or target region has been decided

upon, the nucleotide sequence should be assessed for strong conservation of sequence among the various isolates. Within these conserved regions, the potential cleavage sites, preferably GUC or GUA (others will suffice, but appear to be less efficiently cleaved) should be chosen. The region should be examined for potential secondary structures, and then the most promising sites chosen. Finally, before testing the ribozyme in cell culture, it is advisable to carry out a series of in vitro cleavage reactions (preferably kinetic analyses) using long (at least 100 nucleotides in length) substrates to verify that the chosen sites are truly structurally favorable for cleavage. [Citation omitted.]

Rossi et al. further state that a target which deserves further consideration and testing as a potential ribozyme cleavage site is the viral packaging signal or psi sequence.

Sioud and Drlica, 88 Proc. Natl. Acad. Sci. USA 7303, 1991 describe ribozymes designed to cleave the integrase gene of HIV. They state that when the ribozyme is transcribed from a plasmid in *E. coli* it leads to destruction of the integrase RNA and complete blockage of integrase protein synthesis. They state that the HIV-1 integrase gene may be a useful target for therapeutic ribozymes.

Heidenreich and Eckstein, 267 Journal of Biological Chemistry 1904, 1992, describe three ribozymes targeted to different sites on the long terminal repeat (LTR) RNA of HIV-1. They also describe the influence of chemical modifications within the ribozyme on the cleavage of the LTR RNA, including 2'-Fluorocytidine substitutions and phosphorothioate internucleotidic linkages.

Weerasinghe et al., 65 Journal of Virology 5531, 1991, describe ribozymes designed against a conserved region within the 5' leader sequence of HIV-1 RNA.

Chang et al., 2 Clinical Biotechnology 23, 1990, describe ribozymes designed to target two different sites in the HIV-1 gag gene, and a single site in the viral 5'-LTR region.

5 Lorentzen et al., 5 Virus Genes 17, 1991, describe a ribozyme targeted to the virion infectivity factor (vif) of HIV-1.

Sarver et al., 247 Science 1222, 1990, describe ribozymes in the hammerhead family targeted to HIV-1 gag  
10 transcripts. They state that cells challenged with HIV-1 showed a substantial reduction in the level of HIV-1 gag RNA relative to that in nonribozyme-expressing cells, and that the reduction in gag RNA was reflected by a reduction in antigen p24 levels. They state that the results  
15 suggest the feasibility of developing ribozymes as therapeutic agents against human pathogens such as HIV-1.

Hampel et al., "RNA Catalyst for Cleaving Specific RNA Sequences," filed September 20, 1989, which is a continuation-in-part of U.S. Serial No. 07/247,100, filed  
20 September 20, 1988, describe hairpin ribozymes, and provides an example of such a ribozyme apparently specific to the gag gene of HIV-1. Hampel and Tritz, 28 Biochemistry 4929, 1989 and Hampel et al., 18 Nucleic Acids Research 299, 1990 also describe hairpin catalytic  
25 RNA models and state that one target site is the tat gene in HIV-1.

Goldberg et al., WO 91/04319 and Robertson and Goldberg WO 91/04324, describe ribozymes expressed within a hepatitis delta vector and state that the genome of the  
30 delta virus may carry a ribozyme against the env or gag mRNA of HIV. Rossi et al., WO 91/03162, describe chimeric DNA-RNA catalytic sequences used to cleave HIV-1 gag transcript or the 5' LTR splice site.

Ojwang et al., 89 Proc. Natl. Acad. Sci. USA 10, 802, 1992 and Yu et al., 90 Proc. Natl. Acad. Sci. USA 6340, 1993 describe a hairpin ribozyme allegedly able to inhibit HIV-1 expression. Joseph and Burke 268 J. Biol. Chem. 24,

515 1993, describe optimization of an anti-HIV hairpin ribozyme. Dropulic et al., 66 J. Virology 1432, 1992 describe a U5 ribozyme which cleaves at nucleoside +115 in HIV-1 RNA.

- 5 Other related art includes Rossi et al., U.S. Patents 5,144,019 and 5,149,796; Altman et al., U.S. Patent 5,168,053; Zaia et al., 660 Ann. N.Y. Acad. Sci. 95, 1992; Guatelli et al., 16E J. Cell Biochem. 79, 1992; Jeang et al., 267 J. Biol. Chem. 17891, 1992; Dropulic et al., 66  
10 J. Virol. 1432, 1992; Lisziewicz et al., International Publication WO 91/10453; International Publication WO 91/15500; Rossi et al., 14A J. Cell Biochem. D428, 1990; and "The Papovaviridae", Ed. Salzman et al., Vol. 2, The Viruses, Plenum Press, NY 1987.
- 15 Ribozymes are RNA molecules having an enzymatic activity which is able to repeatedly cleave other separate RNA molecules in a nucleotide base sequence specific manner. Such enzymatic RNA molecules can be targeted to virtually any RNA transcript, and efficient cleavage has  
20 been achieved in vitro. Kim et al., 84 Proc. Natl. Acad. Sci. USA 8788, 1987; Haseloff and Gerlach, 334 Nature 585, 1988; Cech, 260 JAMA 3030, 1988; and Jefferies et al., 17 Nucleic Acids Research 1371, 1989.

Ribozymes act by first binding to a target RNA. Such  
25 binding occurs through the target RNA binding portion of a ribozyme which is held in close proximity to an enzymatic portion of the RNA which acts to cleave the target RNA. Thus, the ribozyme first recognizes and then binds a target RNA through complementary base-pairing, and once  
30 bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After a ribozyme has bound and cleaved its RNA target it is released from that RNA to search for another  
35 target and can repeatedly bind and cleave new targets.

The enzymatic nature of a ribozyme is advantageous over other technologies, such as antisense technology

(where a nucleic acid molecule simply binds to a nucleic acid target to block its translation) since the effective concentration of ribozyme necessary to effect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding, but also on the mechanism by which the molecule inhibits the expression of the RNA to which it binds. That is, the inhibition is caused by cleavage of the RNA target and so specificity is defined as the ratio of the rate of cleavage of the targeted RNA over the rate of cleavage of non-targeted RNA. This cleavage mechanism is dependent upon factors additional to those involved in base pairing. Thus, it is thought that the specificity of action of a ribozyme is greater than that of antisense oligonucleotide binding the same RNA site.

#### Summary of the Invention

The invention features novel enzymatic RNA molecules, or ribozymes, and methods for their use for inhibiting immunodeficiency virus replication, e.g., HIV-1, HIV-2 and related viruses including FIV-1 and SIV-1. Such ribozymes can be used in a method for treatment of diseases caused by these related viruses in man and other animals. The invention also features cleavage of the RNA of these viruses by use of ribozymes. In particular, the ribozyme molecules described are targeted to the *LTR*, *nef*, *vif*, *tat* and *rev* viral genes or regions. These genes are known in the art, see, e.g., Matsukura et al., 86 Proc. Natl. Acad. Sci. USA 4244, 1989; Cheng-Mayer et al., 246 Science 1629, 1989; Viscidi et al., 246 Science 1606, 1989; Malim et al., 86 Proc. Natl. Acad. Sci. USA 8222, 1989; Terwilliger



et al., 88 Proc. Natl. Acad. Sci. USA 10971, 1991; and Bartel et al., 67 Cell 529, 1991, and Figs. 2A and 2B.

Thus, in a first aspect, the invention features an enzymatic RNA molecule (or ribozyme) which cleaves HIV-1 RNA, or its equivalent, regions required for viral replication, e.g., protein synthesis, e.g., the *vif*, *nef*, *tat* or *rev* gene regions, or at structures known to regulate viral gene expression, e.g., *tar*, *rre* or 3'-LTR regions.

By "enzymatic RNA molecule" it is meant an RNA molecule which has complementarity in a substrate binding region to a specified gene target, and also has an enzymatic activity which is active to specifically cleave RNA in that target. That is, the enzymatic RNA molecule is able to intermolecularly cleave RNA and thereby inactivate a target RNA molecule. This complementarity functions to allow sufficient hybridization of the enzymatic RNA molecule to the target RNA to allow the cleavage to occur. One hundred percent complementarity is preferred, but complementarity as low as 50-75% may also be useful in this invention. By "equivalent" RNA to HIV-1 is meant to include those naturally occurring RNA molecules associated with immunodeficiency diseases in various animals, including humans, felines, and simians. These viral RNAs have similar structures and equivalent genes to each other, including the *vif*, *nef*, *tat* and *rev* genes.

By "gene" is meant to refer to either the protein coding regions of the cognate mRNA, HIV genome, proviral genome or any regulatory regions in the RNA which regulate synthesis of the protein or stability of the mRNA.

In preferred embodiments, the enzymatic RNA molecule is formed in a hammerhead motif, but may also be formed in the motif of a hairpin, hepatitis delta virus, group I intron or RNaseP-like RNA (in association with an RNA guide sequence). Examples of such hammerhead motifs are described by Rossi et al. (see citations above), of hairpin motifs by Hampel et al. (see citations above), and an example of the hepatitis delta virus motif is described

by Perrotta and Been, 31 Biochemistry 16, 1992; of the RNaseP motif by Guerrier-Takada, et al., 35 Cell 849, 1983; and of the group I intron by Cech et al., U.S. Patent 4,987,071. These specific motifs are not limiting  
 5 in the invention and those skilled in the art will recognize that all that is important in an enzymatic RNA molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have  
 10 nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule.

In particularly preferred embodiments, the RNA which is cleaved in HIV-1 RNA is selected from one or more of  
 15 the following sequences:

Sequence taken from the HIVPCV12 sequence in the Los Alamos Human Retrovirus and AIDS database. The sequence folded began at nucleotide number one of the 2.3 kb subgenomic mRNA. This region includes the coding regions  
 20 for the vif, vpr, vpu, tat, rev, and nef gene products.

Nucleotide Sequence Number	SEQ.ID.NO.
13 AGAAGAAAAGCAAAGAUCAUUAGGGAUUAUGGAAAACAGA	ID.NO.01
108 AGUUUAGUAAAACAC	ID.NO.02
25 121 CCAUAUGUAUAUJUC	ID.NO.03
198 UCAGAAGUACACAUC	ID.NO.04
228 AGAUUGGUAGUAANA	ID.NO.05
235 AAUAACAACAUUUGG	ID.NO.06
246 AUUGGGGUCUGCAUA	ID.NO.07
30 258 AUACAGGAGAAAGAGACUGGCAUUUGGG	ID.NO.08
280 AUCUGGGUCAGGGAGUCUCCAUA	ID.NO.09
311 AAAAAGAGAUUAGCACACAAGUAGACCCU	ID.NO.10
439 UGAAUAUCAAGCAGGACAUACAAGGUAGGAUCUCUACAAUA	ID.NO.11
468 AUACUUGGCACUAGCAGCAUUAUAACACCAAAAAGAUAAAGC	ID.NO.12
35 601 CACAAUGAAUGGACACUAG	ID.NO.13
644 AAGCUGUUAGA	ID.NO.14
683 UAGGGCAACAUUUCUAUGAAACUUA	ID.NO.15

	733	GCCAUAUAAGAA	ID.NO.16
	800	AUAGGCGUAC	ID.NO.17
	828	GAAAUUGGAGCC	ID.NO.18
	844	AUCCUAGACUAGAGC	ID.NO.19
5	875	AAGUCAGCCUAAAA	ID.NO.20
	894	UGUACCAAUUGCUAUUGUAAAAAGUG	ID.NO.21
	925	UUCAUUGCCAAG	ID.NO.22
	936	GUUUGUUUCAUAACAAAAGCCUUAGGCAUCUCCUAUGGCAGGAA	ID.NO.23
	988	GACAGCGACGAAGAG	ID.NO.24
10	998	AAGACCUCCUCAAG	ID.NO.25
	1011	GGCAGUCAGACUCAUCAAGUUUCUCU	ID.NO.26
	1037	AUCAAAGCAAC	ID.NO.27
	1053	UCCCAAUCCCGAGGGGACCCGACAGGCCCGAAGGAUAGAAGAA	ID.NO.28
	1126	CAUUCGAUUAGUGAA	ID.NO.29
15	1162	GGACGAUCUGCGGAGCCUGUGC	ID.NO.30
	1260	GGGAAGCCCUCAAUAUUGGUGGAAUCUC	ID.NO.31
	1314	AGAAUAGUGCUG	ID.NO.32
	1339	UGCCACAGCUAUAGCA	ID.NO.33
	1383	AAGUAGUACAAGAAGCUUAUAGA	ID.NO.34
20	1419	UACCUAGAAGAAUAAGACAGGGCUUGGAAAGGAU	ID.NO.35
	1476	UGGUCAAAAAGUAG	ID.NO.36
	1517	AAGAAUGAGACGAGCUGAGCCA	ID.NO.37
	1557	GGAGCAGUAUCUCGA	ID.NO.38
	1568	AGACCUAGAAAAACAUGGAGCAAUCACA	ID.NO.39
25	1630	CCUGGCUAGAAGCACAAAGAGGAGGAGAAGGUGGG	ID.NO.40
	1674	ACACCUCAGGUACCUUUAAGACCAAUGACUUACAAG	ID.NO.41
	1710	GCAGCUGUAGAUCUUAGCCACUUUUUAAAAGAAAAGGGGG	ID.NO.42
	1747	GGGGGACUGGAAGGG	ID.NO.43
	1760	GCUAAUUCACUCCCAACGA	ID.NO.44
30	1779	AGACAAGAUAUCCUUGAUCUGUGGAUCUACCACA	ID.NO.45
	1831	AUUGGCAGAACUACACACCAGGAC	ID.NO.46
	1861	UCAGAUAUCCA	ID.NO.47
	1894	AAGCUAGUACCAGUU	ID.NO.48
	1941	GAGAACACCAGCUU	ID.NO.49
35	1960	ACCCUGUGAGCCUGCAUGGAAUGGAUGAC	ID.NO.50
	2008	AGUGGAGGUUUGACAGCCGC	ID.NO.51
	2065	AGUACUUAAGAACUGCUGAUUUCGAGCUUGCUACAAGGGAC	ID.NO.52
	2188	CUGCUUUUUGCCUGUAC	ID.NO.53
	2228	UCUGAGCCUGGGAGCUC	ID.NO.54
40	2281	UAAAGCUUGCC	ID.NO.55

## 3' LTR:

UGCCUGUAGAUCUAGAC

ID.NO.56

AGCAUCCAGGAAGUCAGCC

ID.NO.57

## nef gene:

5	8-23	CAAGUGGUCAAAAANG	ID.NO.58
	93-107	GGAGCAGUAUCUCAA	ID.NO.59
	214-229	CCNCAGGUACCUUUA	ID.NO.60
	283-297	GGGGGACUGGAUGGG	ID.NO.61

## vif gene:

10	80-95	CCAUAUGUAUGUUUC	ID.NO.62
	187-201	AGACUGGUAAUAANA	ID.NO.63
	239-253	AUCUGGGUCAGGGAG	ID.NO.64
		AUUUGGGUCAGGGAG	ID.NO.65
	247-261	CAGGGAGUCUCCAUA	ID.NO.66
15	286-300	ACACAAGUAGACCCU	ID.NO.67
	418-432	AACAAGGUAGGAUCU	ID.NO.68

In a second related aspect, the invention features a mammalian cell which includes an enzymatic RNA molecule as described above. Preferably, the mammalian cell is a human cell, for example, a T4 lymphocyte having a CD4 receptor molecule on its cell surface.

In a third related aspect, the invention features an expression vector which includes nucleic acid encoding the enzymatic RNA molecules described above, located in the vector, e.g., in a manner which allows expression of that enzymatic RNA molecule within a mammalian cell.

In a fourth related aspect, the invention features a method for treatment of human immunodeficiency disease by administering to a patient an enzymatic RNA molecule which cleaves HIV-1 RNA or related RNA in the *vif*, *nef*, *tat* or *rev* gene regions.

In other related aspects, the invention features treatment of cats or simians with ribozymes of this invention. Such ribozymes may be identical to those able to cleave HIV-1 RNA, or may be modified to target analogous locations in FIV and SIV virus RNAs.

The invention provides a class of chemical cleaving agents which exhibit a high degree of specificity for the viral RNA of HIV-1 type virus-infected cells. If desired, such ribozymes can be designed to target equivalent  
5 single-stranded DNAs by methods known in the art. The ribozyme molecule is preferably targeted to a highly conserved sequence region of HIV-1 such that all strains of HIV-1 can be treated with a single ribozyme. Such enzymatic RNA molecules can be delivered exogenously or  
10 endogenously to infected cells. In the preferred hammerhead motif, the small size (less than 40 nucleotides, preferably between 32 and 36 nucleotides in length) of the molecule allows the cost of treatment to be reduced compared to other ribozyme motifs.

15 The smallest ribozyme delivered for treatment of HIV infection reported to date (by Rossi et al., 1992, *supra*) is an *in vitro* transcript having a length of 142 nucleotides. Synthesis of ribozymes greater than 100 nucleotides in length is very difficult using automated methods,  
20 and the therapeutic cost of such molecules is prohibitive. Delivery of ribozymes by expression vectors is primarily feasible using only *ex vivo* treatments. This limits the utility of this approach. In this invention, an alternative approach uses smaller ribozyme motifs (e.g., of the  
25 hammerhead structure, shown generally in Fig. 1) and exogenous delivery. The simple structure of these molecules also increases the ability of the ribozyme to invade targeted regions of the mRNA structure. Thus, unlike the situation when the hammerhead structure is included within  
30 longer transcripts, there are no non-ribozyme flanking sequences to interfere with correct folding of the ribozyme structure, or with its complementary binding of the ribozyme to the mRNA target region.

The enzymatic RNA molecules of this invention can be  
35 used to treat human immunodeficiency virus infections, including those caused by both HIV-1 and HIV-2. Such treatment can also be extended to other related viruses

which infect non-human primates including the simian and feline immunodeficiency viruses. Infected animals can be treated at the time of productive infection. This timing of treatment will reduce viral loads in infected cells and  
5 disable viral replication in any subsequent rounds of infection. This is possible because the ribozymes disable those structures required for successful initiation of viral protein synthesis.

The targets chosen in the present invention provide  
10 a distinct advantage over prior targets since they act not only at the time of viral absorption or reverse transcription during infection, but also in latently infected cells and in virally transformed cells. In addition, viral particles which are released during a first round of  
15 infection in the presence of such ribozymes will still be immunogenic by virtue of having their capsids intact. Thus, one method of this invention allows the creation of defective but immunogenic viral particles, and thus a continued possibility of initiation of an immune response  
20 in a treated animal.

In addition, the enzymatic RNA molecules of this invention can be used in vitro in a cell culture infected with HIV-1 viruses, or related viruses, to produce viral particles which have intact capsids and defective genomic  
25 RNA. These particles can then be used for instigation of immune responses in a prophylactic manner, or as a treatment of infected animals.

The invention also features immunization preparations formed from defective HIV-1 particles (or related  
30 particles) created by a method of this invention, and methods for immunization or vaccination using these defective particles, e.g., with DNA or vectors encoding a ribozyme of this invention under the control of a suitable promoter.

Diagnostic uses

Ribozymes of this invention may be used as diagnostic tools to examine genetic drift and mutations of viruses within diseased cells. The close relationship between  
5 ribozyme activity and the structure of the target RNA allows the detection of mutations in any region of the molecule which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple ribozymes described in this invention, one may  
10 map nucleotide changes which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with ribozymes may be used to inhibit gene expression and define the role (essentially) of specified gene products in the progres-  
15 sion of disease. In this manner, other genetic targets may be defined as important mediators of the disease. These experiments will lead to better treatment of the disease progression by affording the possibility of combinational therapies (e.g., multiple ribozymes targeted  
20 to different genes, ribozymes coupled with known small molecule inhibitors, or intermittent treatment with combinations of ribozymes and/or other chemical or biological molecules). Other in vitro uses of ribozymes of this invention are well known in the art, and include detection  
25 of the presence of mRNA associated with an HIV-1 related condition. Such RNA is detected by determining the presence of a cleavage product after treatment with a ribozyme using standard methodology.

In a specific example, ribozymes which can cleave  
30 only wild-type HIV or mutant forms of the target RNA are used for the assay. The first ribozyme is used to identify wild-type RNA present in the sample and the second ribozyme will be used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of  
35 both wild-type and mutant RNA will be cleaved by both ribozymes to demonstrate the relative ribozyme efficiencies in the reactions and the absence of cleavage of

the "non-targeted" RNA species. The cleavage products from the synthetic substrates will also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus each analysis will  
5 require two ribozymes, two substrates and one unknown sample which will be combined into six reactions. The presence of cleavage products will be determined using an RNase protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a  
10 polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the  
15 phenotype (i.e., HIV) is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels will be adequate and will decrease the cost of the initial diagnosis. Higher mutant form to wild-type ratios will be  
20 correlated with higher risk whether RNA levels are compared qualitatively or quantitatively.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

25 Description of the Preferred Embodiments

The drawings will first briefly be described.

Drawings

Fig. 1 is a diagrammatic representation of a hammer-head motif ribozyme showing stems I, II and III (marked  
30 (I), (II) and (III) respectively) interacting with an HIV-1 target region. The 5' and 3' ends of both ribozyme and target are shown. Dashes indicate base-paired nucleotides.

Figs. 2A and 2B are diagrammatic representations of  
35 the various genes and gene regions in HIV-1 and HIV-2.



Figs. 3A - 3C are diagrammatic representations of three ribozymes of this invention.

5 Figs. 4A - 4G are diagrammatic representations of chemically modified ribozymes of this invention (solid circles indicate modified bases). Specifically, Fig. 4A is unmodified HCH-r37, Fig. 4B is Thio-substituted HCH-r37S2, Fig. 4C is Thio-substituted HCH-r37S4, Figs. 4D - G are Thio substituted HCH-s37A - D.

10 Fig. 5 is a diagrammatic representation of a chemically modified ribozyme of this invention (circled bases are modified with 2'-O-methyl), specifically the ribozyme is 2'-O-methyl HCH-037A.

15 Figs. 6a-6f are graphical representations of ribozyme stability in Vero cell or HeLa cell extracts from cytoplasm, membrane, and nucleus.

Figs. 7, 8 and 9 are diagrammatic representations of various hammerhead ribozymes along with data on activity, and nuclease resistance.

20 Fig. 10 is a graphical representation of activity of various LTR-targeted ribozymes.

Fig. 11 is a graphical representation of activity of ribozymes of various arm lengths.

Fig. 12 is a graphical representation of ribozymes with different sugar modifications.

25 Fig. 13 is a graphical representation of activity of various TAT-targeted ribozymes.

Fig. 14 is a diagrammatic representation of a hammerhead ribozyme showing base numbering. Each N and N' can be the same or different.

30 Fig. 15 is a diagrammatic representation of two hairpin ribozymes active on HIV RNA.

#### Target Sites

The genome of HIV-1 is subject to rapid genetic drift by virtue of its RNA content and the nature of errors in reverse transcription. Those regions (genes) of the genome which are essential for virus replication, however,

35

are expected to maintain a constant sequence (i.e., are conserved) over extensive periods of time. These regions are preferred target sites in this invention since they are more likely to be conserved between different types or strains of immunodeficiency viruses, and thus only one ribozyme is needed to destroy all such viruses. Thus, one ribozyme may be used to target all HIV-1 virus, as well as all HIV-2, SIV and FIV viruses. We have selected several such genes of HIV-1, and examined their nucleotide sequences for the presence of conserved regions which may be cleaved by ribozymes targeted to those regions. Two genes analyzed in detail are the *vif* and *nef* genes; the *tat*, *rev* and other genes noted above can be analyzed in a manner similar to that described below. Nucleotide sequences were acquired from the Los Alamos HIV gene bank.

Ribozymes targeting selected regions of the HIV genome are chosen to cleave the target RNA in a manner which inhibits translation of the RNA. Genes are selected such that inhibition of translation will inhibit viral replication, e.g., by inhibiting protein synthesis. Selection of effective target sites within these critical regions of HIV-1 RNA entails testing the accessibility of the target RNA to hybridization with various oligonucleotide probes. These studies can be performed using RNA probes and assaying accessibility by cleaving the hybrid molecule with RNaseH (see below). Alternatively, such a study can use ribozyme probes designed from secondary structure predictions of the RNAs, and assaying cleavage products by polyacrylamide gel electrophoresis (PAGE), to detect the presence of cleaved and uncleaved molecules.

The following is but one example of a method by which suitable target sites can be identified and is not limiting in this invention. Generally, the method involves identifying potential cleavage sites for a hammerhead ribozyme, and then testing each of these sites to determine their suitability as targets by ensuring that secondary structure formation is minimal.

The HIV-1 genomic sequences of the Los Alamos data bank were compared in the regions encoding the *vif* and *nef* genes. Fifteen putative ribozyme cleavage sites were found to be highly conserved between the 11 strains of virus sequence. These sites represent the preferable sites for hammerhead ribozyme cleavage within these two target RNAs. Two of the *nef* gene sites overlap regions within the 3'-LTR of the HIV-1 genome, which represents another target of potential therapeutic value. All of the *nef* targets are present in all known HIV-1 mRNAs and may represent targets for cleavage which would disrupt 3' terminal control regions of the mRNA which may be required for efficient translation or export of the RNAs. In a similar manner, a number of the *vif* target sites are present in the *pol*, *tat* and *vpr* mRNAs (see Fig. 2).

Short RNA substrates corresponding to each of the *vif* and *nef* gene sites were designed. Each substrate was composed of two to three nucleotides at the 5' and 3' ends that would not base pair with a corresponding ribozyme recognition region. The unpaired regions flanked a central region of 12-14 nucleotides to which complementary arms in the ribozyme were designed.

The structure of each substrate sequence was predicted using a standard commercially available PC fold computer program. Sequences which gave a positive free energy of binding were accepted. Sequences which gave a negative free energy were modified by trimming one or two bases from each of the ends. If the modified sequences were still predicted to have a strong secondary structure, they were rejected.

After substrates were chosen, ribozymes were designed to each of the RNA substrates. Ribozyme folding was also analyzed using PC fold.

Ribozyme molecules were sought which formed hammerhead motif stem II (see Fig. 1) regions and contained flanking arms which were devoid of intramolecular base pairing. Often the ribozymes were modified by trimming a

base from the ends of the ribozyme, or by introducing additional base pairs in stem II to achieve the desired fold. Ribozymes with incorrect folding were rejected. After substrate/ribozyme pairs were found to contain  
 5 correct intramolecular structures, the molecules were folded together to predict intermolecular interactions. A schematic representation of a ribozyme with its coordinate base pairing to its cognate target sequence is shown in Fig. 1.

10 Using such analyses, the following predictions of effective target sites in the *vif* and *nef* genes of the HIV genome, based upon computer generated sequence comparisons, were obtained (see Table 1). The target sequence is listed first with the 5'-most nucleotide number, for  
 15 reference. Bases in parentheses are alternative bases in the conserved patterns.

Table 1

<u>Base numbers</u>		<u>RNA Target sequence</u>
<u>nef gene</u>		
20	8-23	CAAGUGGU C AAAANG
	93-107	GGAGCAGU A UCUCGA (A)
	214-229	CCNCAGGU A CCUJUA
25	283-297	GGGGGACU G GAAGGG (ALSO 3' LTR TARGET) (U)
	430-444	AAGCUAGU A CCAGUU (ALSO 3' LTR TARGET)
<u>vif gene</u>		
	67-81	AGUUUAGU A AAACAC
30	80-95	CCAU AUGU A UAUUUC (G)
	157-171	UCAGAAGU A CACAUC
	187-201	AGAUUGGU A GUAANA (C) (A)
	205-220	AUUGGGGU C UGCAUA

239-253	AUCUGGGU C AGGGAG (U)
247-261	CAGGGAGU C UCCAUA
286-300	ACACAAGU A GACCCU
5 418-432	AACAAGGU A GGAUCU

Those targets thought to be useful as ribozyme targets can be tested to determine accessibility to nucleic acid probes in a ribonuclease H assay (see below). This assay provides a quick test of the use of the target site without requiring synthesis of a ribozyme. It can be used to screen for sites most suited for ribozyme attack.

#### Synthesis of Ribozymes

Ribozymes useful in this invention can be produced by gene transcription as described by Cech, *supra*, or by chemical synthesis. Chemical synthesis of RNA is similar to that for DNA synthesis. The additional 2'-OH group in RNA, however, requires a different protecting group strategy to deal with selective 3'-5' internucleotide bond formation, and with RNA susceptibility to degradation in the presence of bases. The recently developed method of RNA synthesis utilizing the t-butyldimethylsilyl group for the protection of the 2' hydroxyl is the most reliable method for synthesis of ribozymes. The method reproducibly yields RNA with the correct 3'-5' internucleotide linkages, with average coupling yields in excess of 99%, and requires only a two-step deprotection of the polymer.

A method, based upon H-phosphonate chemistry exhibits a relatively lower coupling efficiency than a method based upon phosphoramidite chemistry. This is a problem for synthesis of DNA as well. A promising approach to scale-up of automatic oligonucleotide synthesis has been described recently for the H-phosphonates. A combination of a proper coupling time and additional capping of "failure" sequences gave high yields in the synthesis of

oligodeoxynucleotides in scales in the range of 14  $\mu$ moles with as little as 2 equivalents of a monomer in the coupling step. Another alternative approach is to use soluble polymeric supports (e.g., polyethylene glycols), instead  
5 of the conventional solid supports. This method can yield short oligonucleotides in hundred milligram quantities per batch utilizing about 3 equivalents of a monomer in a coupling step.

Various modifications to ribozyme structure can be  
10 made to enhance the utility of ribozymes. Such modifications will enhance shelf-life, half-life in vitro, stability, and ease of introduction of such ribozymes to the target site, e.g., to enhance penetration of cellular membranes, and confer the ability to recognize and bind to  
15 targeted cells.

Exogenous delivery of ribozymes benefits from chemical modification of the backbone, e.g., by the overall negative charge of the ribozyme molecule being reduced to facilitate diffusion across the cell membrane. The  
20 present strategies for reducing the oligonucleotide charge include: modification of internucleotide linkages by methylphosphonates, use of phosphoramidites, linking oligonucleotides to positively charged molecules, and creating complex packages composed of oligonucleotides,  
25 lipids and specific receptors or effectors for targeted cells. Examples of such modifications include sulfur-containing ribozymes containing phosphorothioates and phosphorodithioates as internucleotide linkages in RNA. Synthesis of such sulfur-modified ribozymes is achieved by  
30 use of the sulfur-transfer reagent,  $^3\text{H}$ -1,2-benzenedithiol-3-one 1,1-dioxide. Ribozymes may also contain ribose modified ribonucleotides. Pyrimidine analogues are prepared from uridine using a procedure employing diethylamino sulphur trifluoride (DAST) as a starting reagent.  
35 Ribozymes can also be either electrostatically or covalently attached to polymeric cations for the purpose of reducing charge. The polymer can be attached to the

ribozyme by simply converting the 3'-end to a ribonucleoside dialdehyde which is obtained by a periodate cleavage of the terminal 2',3'-cis diol system. Depending on the specific requirements for delivery systems, other possible  
5 modifications may include different linker arms containing carboxyl, amino or thiol functionalities. Yet further examples include use of methylphosphonates and 2'-O-methylribose and 5' or 3' capping or blocking with m<sub>7</sub>GpppG or m<sub>3</sub><sup>2,2,7</sup>GpppG.

10 For example, a kinased ribozyme is contacted with guanosine triphosphate and guanyltransferase to add a m<sup>3</sup>G cap to the ribozyme. After such synthesis, the ribozyme can be gel purified using standard procedure. To ensure that the ribozyme has the desired activity, it may be  
15 tested with and without the 5' cap using standard procedures to assay both its enzymatic activity and its stability.

Synthetic ribozymes, including those containing various modifiers, can be purified by high pressure liquid  
20 chromatography (HPLC). Other liquid chromatography techniques, employing reverse phase columns and anion exchangers on silica and polymeric supports may also be used.

There follows an example of the synthesis of one  
25 ribozyme. A solid phase phosphoramidite chemistry was employed. Monomers used were 2'-tert-butyl-dimethylsilyl cyanoethylphosphoramidites of uridine, N-benzoyl-cytosine, N-phenoxyacetyl adenosine, and guanosine (Glen Research, Sterling, VA).

30 Solid phase synthesis was carried out on either an ABI 394 or 380B DNA/RNA synthesizer using the standard protocol provided with each machine. The only exception was that the coupling step was increased from 10 to 12 minutes. The phosphoramidite concentration was 0.1 M.  
35 Synthesis was done on a 1 μmole scale using a 1 μmole RNA reaction column (Glen Research). The average coupling efficiencies were between 97% and 98% for the 394 model,

and between 97% and 99% for the 380B model, as determined by a calorimetric measurement of the released trityl cation.

After synthesis, the blocked ribozymes were cleaved  
5 from the solid support (e.g., CPG), and the bases and diphosphoester moiety deprotected in a sterile vial by incubation in dry ethanolic ammonia (2 mL) at 55°C for 16 hours. The reaction mixture was cooled on dry ice. Later, the cold liquid was transferred into a sterile  
10 screw cap vial and lyophilized.

To remove the 2'-tert-butyl-dimethylsilyl groups from the ribozyme, the obtained residue was suspended in 1 M tetra-n-butylammonium fluoride in dry THF (TBAF), using a 20-fold excess of the reagent for every silyl group, for  
15 16 hours at ambient temperature (about 15-25°C). The reaction was quenched by adding an equal volume of sterile 1 M triethylamine acetate, pH 6.5. The sample was cooled and concentrated on a SpeedVac to half the initial volume.

The ribozymes were purified in two steps by HPLC on  
20 a C4 300 Å 5 µm DeltaPak column in an acetonitrile gradient.

The first step, or "trityl on" step, was a separation of 5'-DMT-protected ribozyme(s) from failure sequences lacking a 5'-DMT group. Solvents used for this step were:  
25 A (0.1 M triethylammonium acetate, pH 6.8) and B (acetonitrile). The elution profile was: 20% B for 10 minutes, followed by a linear gradient of 20% B to 50% B over 50 minutes, 50% B for 10 minutes, a linear gradient of 50% B to 100% B over 10 minutes, and a linear gradient of 100%  
30 B to 0% B over 10 minutes.

The second step was a purification of a completely deblocked ribozyme by a treatment of 2% trifluoroacetic acid on a C4 300 Å 5 µm DeltaPak column in an acetonitrile gradient. Solvents used for this second step were: A  
35 (0.1 M Triethylammonium acetate, pH 6.8) and B (80% acetonitrile, 0.1 M triethylammonium acetate, pH 6.8). The elution profile was: 5% B for 5 minutes, a linear



gradient of 5% B to 15% B over 60 minutes, 15% B for 10 minutes, and a linear gradient of 15% B to 0% B over 10 minutes.

The fraction containing ribozyme, which is in the triethylammonium salt form, was cooled and lyophilized on a SpeedVac. Solid residue was dissolved in a minimum amount of ethanol and ribozyme in sodium salt form was precipitated by addition of sodium perchlorate in acetone. (K<sup>+</sup> or Mg<sup>2+</sup> salts can be produced in an equivalent manner.) The ribozyme was collected by centrifugation, washed three times with acetone, and lyophilized.

#### Expression Vector

While synthetic ribozymes are preferred in this invention, those produced by expression vectors can also be used. In designing a suitable ribozyme expression vector the following factors are important to consider. The final ribozyme must be kept as small as possible to minimize unwanted secondary structure within the ribozyme. A promoter (e.g., the human cytomegalovirus immediate early region (HCMV iel) promoter) should be chosen to be a relatively strong promoter, and expressible both in vitro and in vivo. Such a promoter should express the ribozyme at a level suitable to effect production of enough ribozyme to destroy a target RNA, but not at too high a level to prevent other cellular activities from occurring (unless cell death itself is desired).

A hairpin at the 5' end of the ribozyme is useful to ensure that the required transcription initiation sequence (GG or GGG or GGGAG) does not bind to some other part of the ribozyme and thus affect regulation of the transcription process. The 5' hairpin is also useful to protect the ribozyme from 5'-3' exonucleases. A selected hairpin at the 3' end of the ribozyme gene is useful since it acts as a transcription termination signal, and as a protection from 3'-5' exonuclease activity. One example of a known termination signal is that present on the T7 RNA polymer-

ase system. This signal is about 30 nucleotides in length. Other 3' hairpins of shorter length can be used to provide good termination and RNA stability. Such hairpins can be inserted within the vector sequences to  
5 allow standard ribozymes to be placed in an appropriate orientation and expressed with such sequences attached.

Poly(A) tails are also useful to protect the 3' end of the ribozyme. These can be provided by either including a poly(A) signal site in the expression vector (to  
10 signal a cell to add the poly(A) tail *in vivo*), or by introducing a poly(A) sequence directly into the expression vector. In the first approach, the signal must be located to prevent unwanted secondary structure formation with other parts of the ribozyme. In the second approach,  
15 the poly(A) stretch may reduce in size over time when expressed *in vivo*, and thus the vector may need to be checked over time. Care must be taken in addition of a poly(A) tail which binds poly(A) binding proteins which prevent the ribozyme from acting upon their target  
20 sequence.

#### Ribozyme Testing

Once the desired ribozymes are selected, synthesized and purified, they are tested in kinetic and other experiments to determine their utility. An example of such a  
25 procedure is provided below.

#### Preparation of Ribozyme

Crude synthetic ribozyme (typically 350  $\mu$ g at a time) is purified by separation on a 15% denaturing polyacrylamide gel (0.75 mm thick, 40 cm long) and visualized by UV  
30 shadowing. Once excised, gel slices containing full length ribozyme are soaked in 5 ml gel elution buffer (0.5 M  $\text{NH}_4\text{OAc}$ , 1 mM EDTA) overnight with shaking at 4°C. The eluent is desalted over a C-18 matrix (Sep-Pak cartridges, Millipore, Milford, MA) and vacuum dried. The dried RNA  
35 is resuspended in 50-100  $\mu$ l TE (TRIS 10 mM, EDTA 1 mM, pH

7.2). An aliquot of this solution is diluted 100-fold into 1 ml TE, half of which is used to spectrophotometrically quantitate the ribozyme solution. The concentration of this dilute stock is typically 150-800 nM. Purity of the ribozyme is confirmed by the presence of a single band on a denaturing polyacrylamide gel.

A ribozyme may advantageously be synthesized in two or more portions. Each portion of a ribozyme will generally have only limited or no enzymatic activity, and the activity will increase substantially (by at least 5-10 fold) when all portions are ligated (or otherwise juxtaposed) together. A specific example of hammerhead ribozyme synthesis is provided below.

The method involves synthesis of two (or more) shorter "half" ribozymes and ligation of them together using T4 RNA ligase. For example, to make a 34 mer ribozyme, two 17 mers are synthesized, one is phosphorylated, and both are gel purified. These purified 17 mers are then annealed to a DNA splint strand complementary to the two 17 mers. This DNA splint has a sequence designed to locate the two 17 mer portions with one end of each adjacent each other. The juxtaposed RNA molecules are then treated with T4 RNA ligase in the presence of ATP. Alternatively, the DNA splint strand may be omitted from the ligation reaction if the complementary binding affects favorable ligation of the two RNA molecules. The 34 mer RNA so formed is then HPLC purified.

#### Preparation of Substrates

Approximately 10-30 pmoles of unpurified substrate is radioactively 5' end-labeled with T4 polynucleotide kinase using 25 pmoles of [ $\gamma$ - $^{32}$ P] ATP. The entire labeling mix is separated on a 20% denaturing polyacrylamide gel and visualized by autoradiography. The full length band is excised and soaked overnight at 4°C in 100  $\mu$ l of TE (10 mM Tris-HCl pH 7.6, 0.1 mM EDTA).

### Kinetic Reactions

For reactions using short substrates (between 8 and 16 bases) a substrate solution is made 1X in assay buffer (75 mM Tris-HCl, pH 7.6; 0.1 mM EDTA, 10 mM MgCl<sub>2</sub>) such that the concentration of substrate is less than 1 nM. A ribozyme solution (typically 20 nM) is made 1X in assay buffer and four dilutions are made using 1X assay buffer. Fifteen  $\mu$ l of each ribozyme dilution (i.e., 20, 16, 12, 8 and 4 nM) is placed in a separate tube. These tubes and the substrate tube are pre-incubated at 37°C for at least five minutes.

The reaction is started by mixing 15  $\mu$ l of substrate into each ribozyme tube by rapid pipetting (note that final ribozyme concentrations are 10, 8, 6, 4, 2 nM). Five  $\mu$ l aliquots are removed at 15 or 30 second intervals and quenched with 5  $\mu$ l stop solution (95% formamide, 20 mM EDTA xylene cyanol, and bromphenol blue dyes). Following the final ribozyme time point, an aliquot of the remaining substrate is removed as a zero ribozyme control.

The samples are separated on either 15% or 20% polyacrylamide gels. Each gel is visualized and quantitated with an Ambis beta scanner (Ambis Systems, San Diego, CA).

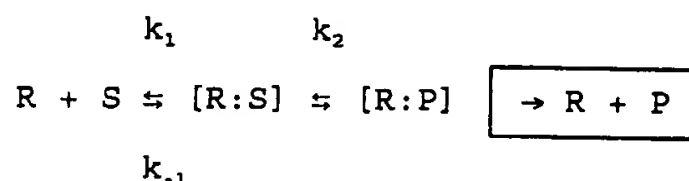
For the most active ribozymes, kinetic analyses are performed in substrate excess to determine  $K_m$  and  $K_{cat}$  values.

For kinetic reactions with long RNA substrates (greater than 15 bases in length) the substrates are prepared by transcription using T7 RNA polymerase and defined templates containing a T7 promoter, and DNA encoding appropriate nucleotides of the HIV-1 RNA. The substrate solution is made 1X in assay buffer (75 mM Tris-HCl, pH 7.6; 0.1 mM EDTA; 10 mM MgCl<sub>2</sub>) and contains 58 nanomolar concentration of the long RNA molecules. The reaction is started by addition of gel purified ribozymes to 1  $\mu$ M concentration. Aliquots are removed at 20, 40, 60, 80 and 100 minutes, then quenched by the addition of 5  $\mu$ l stop solution. Cleavage products are separated using

denaturing PAGE. The bands are visualized and quantitated with an Ambis beta scanner.

### Kinetic Analysis

A simple reaction mechanism for ribozyme-mediated cleavage is:



10

where R = ribozyme, S = substrate, and P = products. The boxed step is important only in substrate excess. Because ribozyme concentration is in excess over substrate concentration, the concentration of the ribozyme-substrate complex ([R:S]) is constant over time except during the very brief time when the complex is being initially formed, i.e., :

$$\frac{d[R:S]}{dt} = 0$$

20 where t = time, and thus:

$$(R)(S)k_1 = (RS)(k_2 + k_{-1}).$$

The rate of the reaction is the rate of disappearance of substrate with time:

$$\text{Rate} = \frac{-d(S)}{dt} = k_2(RS)$$

25

Substituting these expressions:

$$(R)(S)k_1 = 1/k_2 \frac{-d(S)}{dt} (k_2 + k_{-1})$$

or:

30

$$\frac{-d(S)}{S} = \frac{k_1 k_2}{(k_2 + k_{-1})} (R) dt$$

Integrating this expression with respect to time yields:

$$-\ln \frac{S}{S_0} = \frac{k_1 k_2}{(k_2 + k_{-1})} (R) t$$

35 where  $S_0$  = initial substrate. Therefore, a plot of the negative log of fraction substrate uncut versus time (in minutes) yields a straight line with slope:

$$\text{slope} = \frac{k_1 k_2}{(k_2 + k_{-1})} (R) = k_{\text{obs}}$$

5

$$\text{slope} = \frac{k_1 k_2}{(k_2 + k_1)} \text{ which is } \frac{k_{\text{cat}}}{K_m}$$

Using these equations the data obtained from the kinetic experiments provides the necessary information to determine which ribozyme tested is most useful, or active. Such ribozymes can be selected and tested in *in vivo* or *ex vivo* systems.

## 15

Lipid molecules were dissolved in a volatile organic solvent (CHCl<sub>3</sub>, methanol, diethylether, ethanol, etc.). The organic solvent was removed by evaporation. The lipid was hydrated into suspension with 0.1x phosphate buffered saline (PBS), then freeze-thawed 3x using liquid nitrogen and incubation at room temperature. The suspension was extruded sequentially through a 0.4 μm, 0.2 μm and 0.1 μm polycarbonate filters at maximum pressure of 800 psi. The ribozyme was mixed with the extruded liposome suspension and lyophilized to dryness. The lipid/ribozyme powder was rehydrated with water to one-tenth the original volume. The suspension was diluted to the minimum volume required for extrusion (0.4 ml for 1.5 ml barrel and 1.5 ml for 10 ml barrel) with 1xPBS and re-extruded through 0.4 μm, 0.2 μm, 0.1 μm polycarbonate filters. The liposome entrapped ribozyme was separated from untrapped ribozyme by gel filtration chromatography (SEPHAROSE CL-4B, BIOGEL A5M). The liposome extractions were pooled and sterilized by filtration through a 0.2 μm filter. The free ribozyme was pooled and recovered by ethanol precipitation. The liposome concentration was determined by incorporation of a radioactive lipid. The ribozyme concentration was determined by labeling with <sup>32</sup>P. Rossi et al., 1992, *supra* (and references cited therein), describe other methods suitable for preparation of liposomes.

In experiments with a liposome formulation composed of a synthetic lipid derivative disteraoyl-phosphatidyl-ethylamidothioacetyl succinimide (DSPE-ATS) co-formulated with dipalmitoylphosphatidyl choline and cholesterol we  
5 observed uptake of 100 and 200 nm diameter liposomes with similar kinetics. The larger particles accommodated a larger number of entrapped molecules, or larger molecular weight molecules, such as an expression plasmid. These particles showed a linear relationship between the lipid  
10 dose offered and the mean log fluorescence (calcein was used to follow liposome uptake). No cytotoxicity was observed even with a 200  $\mu$ M dose. These liposomes are particularly useful for delivery to CD4 cell populations.

#### In Vivo Assay

15 The efficacy of action of a chosen ribozyme may be tested in vivo by use of cell cultures sensitive to HIV-1 or a related virus, using standard procedures. For example, monolayer cultures of HIV-sensitive cells are grown by established procedures. Cells are grown in 6 or  
20 96 well tissue culture plates. Prior to infection with HIV, cultures are treated for 3 to 24 hours with ribozyme-containing liposomes. Cells are then rinsed with phosphate buffered saline (PBS) and virus added at a multiplicity of 1-100 pfu/cell. After a one-hour adsorption,  
25 free virus is rinsed away using PBS, and the cells are treated for three to five days with appropriate liposome preparations. Cells are then re-fed with fresh medium and re-incubated. Virus is harvested from cells into the overlying medium. Cells are broken by three cycles of  
30 incubation at  $-70^{\circ}\text{C}$  and  $37^{\circ}\text{C}$  for 30 minutes at each temperature, and viral titers determined by plaque assay using established procedures.

#### Ribonuclease Protection Assay

The accumulation of target mRNA in cells or the  
35 cleavage of the RNA by ribozymes or RNaseH (in vitro or

*in vivo*) can be quantified using an RNase protection assay.

In this method, antisense riboprobes are transcribed from template DNA using T7 RNA polymerase (U.S. Biochemical) in 20  $\mu$ l reactions containing 1X transcription buffer (supplied by the manufacturer), 0.2 mM ATP, GTP and UTP, 1 U/ $\mu$ l pancreatic RNase inhibitor (Boehringer Mannheim Biochemicals) and 200  $\mu$ Ci  $^{32}$ P-labeled CTP (800 Ci/mmol, New England Nuclear) for 1 hour at 37°C. Template DNA is digested with 1 U RNase-free DNaseI (U.S. Biochemical, Cleveland, OH) at 37°C for 15 minutes and unincorporated nucleotides removed by G-50 SEPHADEX spin chromatography.

In a manner similar to the transcription of antisense probe, the target RNA can be transcribed *in vitro* using a suitable DNA template. The transcript is purified by standard methods and digested with ribozyme at 37°C according to methods described later.

Alternatively, virus-infected cells are harvested into 1 ml of PBS, transferred to a 1.5 ml EPPENDORF tube, pelleted for 30 seconds at low speed in a microcentrifuge, and lysed in 70  $\mu$ l of hybridization buffer (4 M guanidine isothiocyanate, 0.1% sarcosyl, 25 mM sodium citrate, pH 7.5). Cell lysate (45  $\mu$ l) or defined amounts of *in vitro* transcript (also in hybridization buffer) is then combined with 5  $\mu$ l of hybridization buffer containing  $5 \times 10^5$  cpm of each antisense riboprobe in 0.5 ml Eppendorf tubes, overlaid with 25  $\mu$ l mineral oil, and hybridization accomplished by heating overnight at 55°C. The hybridization reactions are diluted into 0.5 ml RNase solution (20 U/ml RNaseA, 2 U/ml RNaseT1, 10 U/ml RNase-free DNaseI in 0.4 M NaCl), heated for 30 minutes at 37°C, and 10  $\mu$ l of 20% SDS and 10  $\mu$ l of Proteinase K (10 mg/ml) added, followed by an additional 30 minutes incubation at 37°C. Hybrids are partially purified by extraction with 0.5 ml of a 1:1 mixture of phenol/chloroform; aqueous phases are combined with 0.5 ml isopropanol, and RNase-resistant hybrids



pelleted for 10 minutes at room temperature (about 20°C) in a microcentrifuge. Pellets are dissolved in 10  $\mu$ l loading buffer (95% formamide, 1X TBE, 0.1% bromophenol blue, 0.1% xylene cyanol), heated to 95°C for five minutes, cooled on ice, and analyzed on 4% polyacrylamide/7 M urea gels under denaturing conditions.

#### Ribozyme Stability

The chosen ribozyme can be tested to determine its stability, and thus its potential utility. Such a test can also be used to determine the effect of various chemical modifications (e.g., addition of a poly(A) tail) on the ribozyme stability and thus aid selection of a more stable ribozyme. For example, a reaction mixture contains 1 to 5 pmoles of 5' (kinased) and/or 3' labeled ribozyme, 15  $\mu$ g of cytosolic extract and 2.5 mM MgCl<sub>2</sub> in a total volume of 100  $\mu$ l. The reaction is incubated at 37°C. Eight  $\mu$ l aliquots are taken at timed intervals and mixed with 8  $\mu$ l of a stop mix (20 mM EDTA, 95% formamide). Samples are separated on a 15% acrylamide sequencing gel, exposed to film, and scanned with an Ambis.

A 3'-labeled ribozyme can be formed by incorporation of the <sup>32</sup>P-labeled cordycepin at the 3' OH using poly(A) polymerase. For example, the poly(A) polymerase reaction contains 40 mM Tris, pH 8, 10 mM MgCl<sub>2</sub>, 250 mM NaCl, 2.5 mM MnCl<sub>2</sub>; 3  $\mu$ l P<sup>32</sup> cordycepin, 500 Ci/mM; and 6 units poly(A) polymerase in a total volume of 50  $\mu$ l. The reaction mixture is incubated for 30 minutes at 37°C.

#### Effect of Liposome Surface Modifications on Lymphocyte and Macrophage Uptake

Liposomes containing distearoylphosphatidyl ethanolamidomethyl thioacetate can be prepared. The thiol group can be deprotected using hydroxylamine and the reactive thiol can then be modified with thiol reactive groups to alter the surface properties of the liposomes. Reaction with N-ethylmaleimide leads to lymphocyte and macrophage

uptake. This modification results in liposome uptake by CD4<sup>+</sup> and CD4<sup>+</sup> lymphocytes.

Modification with iodoacetamide and iodoacetic acid were tested for uptake. Iodoacetamide modification showed  
5 that the liposomes were toxic in that the lymphocyte cell number decreased with time. However, the acetate uptake was 5-fold greater than that observed for succinimide. Uptake was monitored by both uptake of a <sup>3</sup>H-hexadecyl-cholesterol ether and an entrapped water soluble  
10 fluorophore, calcein. Both confirmed that the liposomes were being taken up by the cells over a 72 hour period. The uptake was assayed in lymphoblasts which had been stimulated for 72 hours with phytohemagglutinin. The uptake curve was biphasic in that within the first 8  
15 hours, only cell surface binding was observed whereas after 24 hours, uptake was linear for up to 72 hours. This was observed for a 200  $\mu$ M dose. The 100  $\mu$ M lipid dose showed only cell surface binding.

#### Effect of Base Substitution Upon Ribozyme Activity

20 To determine which primary structural characteristics could change ribozyme cleavage of substrate, minor base changes can be made in the substrate cleavage region recognized by a specific ribozyme. For example, the substrate sequences can be changed at the central "C"  
25 nucleotide, changing the cleavage site from a GUC to a GUA motif. The  $K_{cat}/K_m$  values for cleavage using each substrate are then analyzed to determine if such a change increases ribozyme cleavage rates. Similar experiments can be performed to address the effects of changing bases complementary to the ribozyme binding arms. Changes predicted  
30 to maintain strong binding to the complementary substrate are preferred. Minor changes in nucleotide content can alter ribozyme/substrate interactions in ways which are unpredictable based upon binding strength alone.  
35 Structures in the catalytic core region of the ribozyme recognize trivial changes in either substrate structure or

the three dimensional structure of the ribozyme/substrate complex.

To begin optimizing ribozyme design, the cleavage rates of ribozymes containing varied arm lengths, but  
5 targeted to the same length of short RNA substrate can be tested. Minimal arm lengths are required and effective cleavage varies with ribozyme/substrate combinations.

The cleavage activity of selected ribozymes can be assessed using HIV-1-homologous substrates. The assays  
10 are performed in ribozyme excess and approximate  $K_{cat}/K_{min}$  values obtained. Comparison of values obtained with short and long substrates indicates utility *in vivo* of a ribozyme.

#### Intracellular Stability of Liposome-delivered Ribozymes

15 To test the stability of a chosen ribozyme *in vivo* the following test is useful. Ribozymes are  $^{32}\text{P}$ -end-labeled, entrapped in liposomes and delivered to HIV-1 sensitive cells for three hours. The cells are fractionated and ribozyme is purified by phenol/chloroform  
20 extraction. Alternatively, cells ( $1 \times 10^7$ , T-175 flask) are scraped from the surface of the flask and washed twice with cold PBS. The cells are homogenized by douncing 35 times in 4 ml of TSE (10 mM Tris, pH 7.4, 0.25 M Sucrose, 1 mM EDTA). Nuclei are pelleted at 100xg for 10 minutes.  
25 Subcellular organelles (the membrane fraction) are pelleted at 200,000xg for two hours using an SW60 rotor. The pellet is resuspended in 1 ml of H buffer (0.25 M Sucrose, 50 mM HEPES, pH 7.4). The supernatant contains the cytoplasmic fraction (in approximately 3.7 ml). The  
30 nuclear pellet is resuspended in 1 ml of 65% sucrose in TM (50 mM Tris, pH 7.4, 2.5 mM  $\text{MgCl}_2$ ) and banded on a sucrose step gradient (1 ml nuclei in 65% sucrose TM, 1 ml 60% sucrose TM, 1 ml 55% sucrose TM, 50% sucrose TM, 300  $\mu\text{l}$  25% sucrose TM) for one hour at 37,000xg with an SW60  
35 rotor. The nuclear band is harvested and diluted to 10% sucrose with TM buffer. Nuclei are pelleted at 37,000xg

using an SW60 rotor for 15 minutes and the pellet resuspended in 1 ml of TM buffer. Aliquots are size fractionated on denaturing polyacrylamide gels and the intracellular localization determined. By comparison to  
5 the migration rate of newly synthesized ribozyme, the various fraction containing intact ribozyme can be determined.

To investigate modifications which would lengthen the half-life of ribozyme molecules intracellularly, the cells  
10 may be fractionated as above and the purity of each fraction assessed by assaying enzyme activity known to exist in that fraction.

The various cell fractions are frozen at -70°C and used to determine relative nuclease resistances of modified ribozyme molecules. Ribozyme molecules may be  
15 synthesized with 5 phosphorothioate (ps), or 2'-O-methyl (2'-OMe) modifications at each end of the molecule. These molecules and a phosphodiester version of the ribozyme are end-labeled with <sup>32</sup>P and ATP using T4 polynucleotide  
20 kinase. Equal concentrations are added to the cell cytoplasmic extracts and aliquots of each taken at 10 minute intervals. The samples are size fractionated by denaturing PAGE and relative rates of nuclease resistance analyzed by scanning the gel with an Ambis β-scanner. The  
25 results show whether the ribozymes are digested by the cytoplasmic extract, and which versions are relatively more nuclease resistant. Modified ribozymes generally maintain 80-90% of the catalytic activity of the native ribozyme when short RNA substrates are employed.

30 Unlabeled, 5' end-labeled or 3' end-labeled ribozymes can be used in the assays. These experiments can also be performed with human cell extracts to verify the observations.

HeLa (CD4<sup>+</sup>) and Vero cells are used by many investigators to screen antiviral chemicals in pre-clinical  
35 trials. Ribozymes and antisense DNAs depend upon their nuclease stability to maintain therapeutic levels in

infected cells. To design chemical modifications which increase the nuclease stability of ribozymes, we have prepared nuclear, cytoplasmic and membranous lysates from various cell types and compared the relative specific activity (units of nuclease/mg protein) and cation optima ( $Mg^{+2}$ -,  $Mn^{+2}$ -,  $Ca^{+2}$ - or  $Zn^{+2}$ -activation) of the nuclease populations within these fractions. Nuclease activities vary significantly among the cell fractions studied (Tables 2 and 3, and Figs. 6a-6f; +++ indicates high nuclease activity, ++ and + indicate lower levels, and - indicates no nuclease activity as tested by standard methodology), but the nuclease population present in HeLa cells was dissimilar to that observed in both monocyte and lymphocyte preparations (Table 4, standard units are noted in the table for comparison only and are not reflective of a specific level of nuclease activity).

Table 2Relative Activity of Cytoplasmic Extracts

<u>Cell Type</u>	<u>Units/10ug of Protein</u>	<u>Divalent cation Requirement</u>
20 Vero	1	Mg <sup>++</sup>
HeLa	5.8	Zn <sup>++</sup>
Cervical Epithelial	4.7	Zn <sup>++</sup>
Monocyte enriched	12.9	none
T Lymphocytes	23.5	none
25 Keratinocytes	4.8	Zn <sup>++</sup>

Table 3Divalent cation effect on RNA degradative activity in HeLa fractions

		<u>Divalent cation (1 mM)</u>			
<u>HeLa cell fractions</u>		<u>MgCl<sub>2</sub></u>	<u>MnCl<sub>2</sub></u>	<u>CaCl<sub>2</sub></u>	<u>ZnCl<sub>2</sub></u>
30	Cytoplasmic	+	+	+	+++
	Membrane	+++	+++	+	-
	Nuclear	+++	++	+++	++

Table 4  
Divalent cation effect on RNA  
degradative activity in Vero fractions

		<u>Divalent cation (1 mM)</u>			
<u>Vero cell fractions</u>		<u>MgCl<sub>2</sub></u>	<u>MnCl<sub>2</sub></u>	<u>CaCl<sub>2</sub></u>	<u>ZnCl<sub>2</sub></u>
5	Cytoplasmic	+++	+++	-	-
	Membrane	+++	+++	-	-
	Nuclear	+++	+++	++	-

HeLa nucleases were maximally active in the presence of added  $Zn^{+2}$ , while added cation did not enhance nuclease activities in monocyte and lymphocyte lysates. The relative nuclease levels in the monocyte and lymphocyte lysates were 2.2 and 4 times higher, respectively, than in activated HeLa lysates.

Various chemical modifications to ribozymes were tested for their ability to increase nuclease activity while maintaining catalytic activity of the ribozyme. Fig. 7 shows the effects of various modifications upon the catalytic activity of a selected ribozyme. The modifications which decreased activity (1315, 1371, and 1285) were dropped from further analyses. Chemical modifications to ribozymes resulted in cell-specific patterns of ribozyme stability, and 2'-O-methyl sugar substitution gave the best overall enhancement of ribozyme stability across lysates. Relative resistance of ribozymes to digestion by lymphocyte and monocyte cytoplasmic lysates are shown in Fig. 8. The digestion rates of the ribozymes are similar when the ribozymes are labeled at either the 5'- or 3'-ends of the molecule, demonstrating that the degradation is not due to endogenous phosphatase activity in the lysates.

Various reports have suggested that modifications to hammerhead motif ribozymes in the binding arms (stems I and III) will give much enhanced protection from nuclease digestion. As shown in Fig. 9, we observe different results. There appear to be nuclease resistant sites at

the positions 2.4, 2.5, 7, 8, 9, 10.4, and 13. The 2.4, 2.5 and 10.4 position resistance may be specific to the ribozyme tested and more sequences need to be tested before a conclusion can be reached concerning the nuclease sensitivity of these sites, but the positions 7, 8, 9 and 13 are conserved nucleotides in this motif and appear to be sites at which nuclease digestion stops, as demonstrated by the appropriate stable fragments produced in lysate digestion experiments. Interestingly, although there seems to be much more nuclease in the lymphocyte and monocyte lysates, no stable fragments were observed when similar experiments were performed using an activated HeLa cytoplasmic lysate. Observations of ribozyme efficacy in HeLa CD4<sup>+</sup> cells should be re-examined in lymphocytes before the therapeutic relevance can be determined.

#### Example 1: HIV tat Ribozymes

The 5' exon of tat contains the following potential cleavage sites: 2 GUC sites, 3 GUA sites, 5 AUC sites, 3 UCC sites, and 5 CUC sites. All 18 sites were examined by computer folding and by RNaseH cleavage assay.

A measure of the accessibility of each site to binding by a 13-mer oligonucleotide was preferred in the following way:

(a) The first 425 nucleotides of the clone V sequence (this clone was made available by Dr. Rossi and includes the 5' tat exon at nucleotides 151-366) was folded on RNAFOLD 4.0 (a generally available program) and examined for the presence of folding domains, i.e., self-contained structures closed by a stem.

(b) For each potential cleavage site, the domain containing that site was folded to confirm that it folded as in part (a); then the domain was refolded while forcing the cleavage site and surrounding nucleotides (11-16 nucleotides in all) to remain unpaired. The difference in these two folding free energies was taken as the cost of

melting out that region for base-pairing by a ribozyme or DNA oligonucleotide.

(c) The lengths of DNA oligonucleotides were adjusted to give predicted delta-G (binding) of -17 to -18 kcal/mole. Thus, differences in overall binding energy was predicted to be reflected in the free energy differences calculated in part (b). The calculations are shown in Table 5.

Table 5

		Delta G			Delta G	
Total		Melting			Binding	Delta G
Start						
<u>Seq</u>	<u># Site</u>	<u>Structure</u>	<u>Length</u>	<u>Oligo</u>	<u>Binding</u>	
194	GUC 1 (@200)	+ 8.6	11	-17.2	- 8.6	
332	GUC 2 (@338)	+ 8.2	11	-17.3	- 9.1	
15 156	GUA 3 (@163)	+ 7.5	12	-17.7	-10.2	
211	GUA 4 (@218)	+ 8.0	13	-17.5	- 9.7	
225	GUA 5 (@233)	+ 6.8	15	-16.3	- 9.5	
161	AUC 6 (@167)	+ 5.4	13	-18.0	-12.6	
186	AUC 7 (@192)	+ 1.6	11	-17.7	-16.1	
20 280	AUC 8 (@286)	+ 5.5	11	-17.9	-12.4	
340	AUC 9 (@347)	+ 7.1	13	-17.0	- 9.9	
352	AUC 10 (@360)	+ 1.7	14	-17.4	-15.7	
240	UUC 11 (@248)	+11.7	14	-17.4	- 5.7	
257	UUC 12 (@265)	+10.7	16	-17.4	- 6.7	
25 346	UUC 13 (@354)	+ 9.7	14	-17.2	- 7.5	
281	CUC 14 (@288)	+ 5.5	12	-17.6	-12.1	
319	CUC 15 (@326)	+11.9	11	-17.1	- 5.2	
322	CUC 16 (@329)	+13.8	11	-17.5	- 3.7	
337	CUC 17 (@344)	+ 6.3	12	-17.2	-10.9	
30 349	CUC 18 (@356)	+10.6	14	-17.2	- 6.6	

[site refers to nucleotide 5' of cleaved phosphate]

Eighteen DNA oligonucleotides were made to target the 18 target sites listed in Table 5. RNaseH experiments were performed using approximately 100 nM body labeled RNA



transcript (clone V), 0.08 U/ $\mu$ l RNaseH (excess RNaseH) and a 2x dilution of 1 mM, 10  $\mu$ M or 1  $\mu$ M DNA oligonucleotide. The results are shown in Table 5. Eight out of 18 sites had greater than 40% cleavage at 5  $\mu$ M after 10 minutes incubation. Three ribozymes were designed against the three most active sequences (H332, H337b, H352, see Table 6).

Table 6

Oligo Name	Sequence (5'→3')	Core	Length	Binding	ENERGIES		1mM/30"	% CLEAVAGE	
					Melting	Net		10μM/10"	1μM/10"
H156	GATCTACTGGCT	GUA	12	-18.5	7.5	-11	83	36	0
H161	GTCTAGGATCTAC	AUC	13	-18.8	5.4	-13.4	12	0	0
H186	TCCTGGATGCT	AUC	11	-18.3	9.4	-8.9	82	0	0
H194b	GGCTGACTTCC	GUC	11	-18.6	8.6	-10	99	84	0
H211v2	ATTGGTACAAGCA	GUA	13	-18	8	-10	61	21	0
H225	ACTTTTACAAATAGC	GUA	15	-17	6.8	-10.2	47	0	0
H240	GCAATGAAAGCAAC	UUC	14	-19.4	11.7	-7.7	25	0	0
H257	TTGTTATGAAACAAAC	UUC	16	-18	10.7	-7.3	100	0	0
H280	AGGAGATGCCT	AUC	11	-18.2	5.5	-12.7	96	60	0
H281	ATAGGAGATGCC	CUC	12	-18.5	6.3	-12.2	84	11	0
H319	GAGGAGGTCTT	CUC	11	-17.4	11.9	-5.5	87	40	0
H322	CTTGAGGAGGT	CUC	11	-16.9	13.8	-3.1	94	8	0
H332	GTCTGACTGCC	GUC	11	-18.7	8.2	-10.5	94	88	0
H337b	TGATGAGTCTGA	CUC	12	-17.6	6.3	-11.3	100	99	80
H340v2	AACCTTGATGAGTC	AUC	13	-17.4	7.1	-10.3	99	66	0
H346	ATAGAGAACTTGA	UUC	14	-17.2	9.7	-7.5	69	10	0
H349	TTGATAGAGAAACT	CUC	14	-17.2	10.6	-6.6	55	23	0
H352	GCTTTGATAGAGAA	AUC	14	-18.5	1.7	-16.8	94	100	84

5

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These three ribozymes are shown in Figs. 3A, B and C, labeled respectively HDH, HEH and HFH.

Example 2: Ribozymes Containing Thiophosphate

The purpose of this example was to evaluate the activity of ribozymes containing substitutions of thiophosphate for phosphate at some backbone positions.

Ribozymes to the HIV-1 tat gene were synthesized on an ABI synthesizer using standard phosphoramidite chemistry, however, at steps where thiophosphate was to be incorporated in the backbone the standard oxidation step (involving Iodine) was replaced with an oxidation step utilizing the Beaucage sulfur transfer reagent. Deprotected and desalted RNA was gel purified, eluted, kinased and sequenced to ensure that the sequence was correct. The end-labeled RNA was also treated with  $H_2O_2$  which preferentially promotes cleavage at positions containing thiophosphate.

Ribozyme activity was tested against cleavage of a short (12 nucleotides) end-labeled substrate RNA. Substrate concentration was approximately 1 nM; ribozyme concentration was 5-100 nM; incubation was at 37°C in 75 mM Tris (pH 7.5), 0.1 mM EDTA, 10 mM  $MgCl_2$  for 2-40 minutes. Cleavage extents were determined by gel electrophoresis (PAGE) followed by quantitation on the AMBIS.

The ribozymes are shown in Figs. 4A - 4G. The following ribozymes showed essentially 100% activity: r37 (unmodified), r37s2 (1 thio on 5' arm, 2 on 3' arm), r37s4 (3 thio modifications on each arm), s37A (3 modifications on 3' arm), s37B (3 modifications alternating on 3' arm).

Ribozymes that are fully modified showed some decrease in activity, for example, s37C (almost completely modified on substrate binding arms) showed a 3x reduction in activity relative to unmodified, s37D (thio modifications in stem I, II and III) showed about a 9x reduction in activity relative to unmodified.

Example 3: 2'-O-methyl-containing Ribozymes

Ribozymes were made on an ABI synthesizer using standard phosphoramidite chemistry with 2'-O-methyl phosphoramidite nucleotides used in place of standard  
5 nucleotides. Ribozyme activity was determined in the manner described above with PAGE analysis.

Referring to Fig. 5, a ribozyme containing 2'-O-methyl at all positions that base-pair with substrate (except for the A at the 5' side of stem III) was synthesized. The  $K_{cat}/K_m$  for the 2'-O-methyl ribozyme was  $44 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$  compared to  $41 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$  for the unmodified, and  $32 \times 10^6$  for a thiophosphate modified ribozyme. Thus, the 2'-O-methyl ribozyme retains 100% activity.

15 Example 4: Targeting the LTR and TAT Regions of HIV-1

The following example extends those provided above to show useful ribozymes targeted to the LTR and TAT regions of HIV-1. Details of methodology used herein are provided in Stinchcomb et al., Methods and Compositions for  
20 Treatment of Restenosis and Cancer Using Ribozymes, USSN 08/245,466, filed 5/18/94 hereby incorporated by reference herein. Such details are not required to practice the invention. Numbering of bases is according to GenBank Nos. K03455 (HIVHXB2) (numbered from transcription start  
25 site).

Screening LTR region for HH Ribozyme Sites:

The LTR is among the most conserved regions within the HIV-1 genome. Also, the LTR region is present in all the transcripts generated during HIV-1 life cycle. So,  
30 ribozymes that cleave LTR targets will potentially block HIV replication.

There are 43 potential hammerhead (HH) ribozyme sites within the HIV-1 LTR. Ten hammerhead ribozymes were synthesized based on a) proper folding of the ribozyme

with its target and b) conservation of target sequence among all HIV-1 strains.

#### RNA Synthesis:

Ribozymes with 7/7 binding arms were synthesized using RNA phosphoramidite chemistry. Ribozymes were deprotected and purified as described above.

Target RNA used in this study was 613 nt long and contained cleavage sites for all the 10 HH ribozymes targeted against LTR. A template containing T7 RNA polymerase promoter upstream of LTR target sequence, was PCR amplified from an HIV-1 pro-DNA clone. Other such clones can be readily constructed. Target RNA was transcribed from this PCR amplified template using T7 RNA polymerase. The transcript was internally labeled during transcription by including [ $\alpha$ - $^{32}$ P] CTP as one of the four ribonucleotide triphosphates. The transcription mixture was treated with DNase-1, following transcription at 37°C for 2 hours, to digest away the DNA template used in the transcription. RNA was precipitated with isopropanol and the pellet was washed two times with 70% ethanol to get rid of salt and nucleotides used in the transcription reaction. RNA is resuspended in DEPC-treated water and stored at 4°C.

#### Ribozyme Cleavage Reactions:

Reactions were carried out under ribozyme excess ( $k_{cat}/K_m$ ) conditions (Herschlag and Cech (1990) Biochemistry 29, 10159-10171). Briefly, 1,000 nM ribozyme and 10 nM internally labeled target RNA were denatured separately by heating to 90°C for 2 min in the presence of 50 mM Tris.HCl, pH 7.5 and 10 mM MgCl<sub>2</sub>. The RNAs were renatured by cooling to 37°C for 10-20 min. Cleavage reaction was initiated by mixing the ribozyme and target RNA at 37°C. Aliquots of 5  $\mu$ l were taken at regular intervals of time and the reaction was quenched by adding equal volume of

stop buffer and freezing on dry ice. The samples were resolved on sequencing gel.

Results:

As shown in Fig. 10, of the ten ribozymes that were tested individually, only four HH ribozymes (568, 581, 631, 696) cleaved the LTR RNA. Other target sites appear to be inaccessible to ribozyme binding and cleavage. Site 568 is also referred to as site 115 and has been targeted for hammerhead ribozyme cleavage by Drouplic et al., 66 J. Virol. 1432-1441, 1992 and Heidenreich and Eckstein, 267 J. Biol. Chem. 1904-1909, 1992.

Since 568 HH ribozyme was cleaving its target to a greater extent than the others, we were interested in optimizing the length of binding arms of this HH ribozyme. As shown in Fig. 11, the rate of ribozyme cleavage increased significantly when the length of the binding arm was increased from 12 to 14 base pairs (total). There was no significant improvement in the activity of ribozymes with binding arms longer than 14 base pairs.

Chemical modification of HH ribozymes targeted to a specific site can significantly improve the stability of the ribozyme in human serum. Further, these modifications do not seem to have any significant effect on the catalytic activity of the ribozyme. All the 2' hydroxyl groups within the ribozyme, with the exception of positions U4, G5, A6, U7, G8, G12 and A15.1 (using standard nomenclature, See Fig. 14), were modified with 2'-O-methyl groups. The 2' hydroxyl groups at U4 and U7 were modified with either 2'-amino, 2'-C-allyl, 2'-O-methyl or 2'-ara-flouro. See Usman et al., 2'-Deoxy 2'-alkylnucleotide containing Nucleic Acid, USSN 08/218,934, filed March 29, 1994, hereby incorporated by reference herein.

Referring to Fig. 12, the 568 HH ribozyme could be stabilized by chemical modification of the sugar moiety of various bases (similar to the ones listed above). The 568

HH ribozyme was extensively modified with one of the following compounds: 2'fluoro, 2'amino at the U7 position, arafluoro at U4 position, 2'amino at U4 and U7 positions, c-allyl at U4 position. None of the above modifications  
5 had any deleterious effect on the ribozyme activity.

Transfection of cells with 568 HH ribozyme (with U4 and U7 positions containing 2'amino modifications) blocks the replication of HIV-1 replication.

Screening TAT region for HH Ribozyme Sites:

10 A region of the TAT mRNA is present in the majority of the transcripts generated during HIV-1 life cycle. So, ribozymes that cleave TAT targets will potentially block HIV replication.

There are 54 potential HH ribozyme site within the  
15 HIV-1 TAT regions (between 5776 nt and 6044 nt). Nine hammerhead ribozymes were synthesized based on a) proper folding of the ribozyme with its target and b) conservation of target sequence among all HIV-1 strains.

RNA synthesis and ribozyme cleavage reactions were  
20 carried out as described above. Target RNA used in this study was 422 nt long.

As shown in Fig. 13, five sites (5841, 5869, 5878, 5966, 5969) are cleaved more readily than the others. None of these hammerhead sites have previously been  
25 targeted.

Screening HIV-1 genome for Hairpin Ribozyme Sites:

Referring to Table VIII and Fig. 15, there are 27 potential hairpin (HP) ribozyme sites in the HIV-1 genome. Ribozymes shown in the table were synthesized and tested.  
30 Modifications to various regions of the hairpin structure can be made without deleterious effect, e.g., in those targetted to n.t. positions 565 and 4398 two extra bases can be inserted in place of GGCA (3rd col.) to GGCACA; and GCAG to GCAGUC respectively.

Site 565 within the LTR region (Ojwang et al., (1992) PNAS. USA. 89, 10802-10806; Yu et al., (1993) PNAS. USA. 90, 6340-6344) and 4398 within the POL region of the HIV-1 genome (Joseph and Burke (1993) J. Biol. Chem. 268, 24515-24518) have been shown to be accessible to HP ribo-  
5 zyme binding and cleavage. We have also found that HP ribozymes targeted towards 565 and 4398 sites are active.

The best HH and HP ribozymes are shown in Table VII, with their associated cleavage and target sites.

#### 10 Administration of Ribozyme

Selected ribozymes can be administered prophylactically, or to HIV-1 infected patients, e.g., by exogenous delivery of the ribozyme to an infected tissue by means of an appropriate delivery vehicle, e.g., a liposome, a  
15 controlled release vehicle, by use of iontophoresis, electroporation or ion paired molecules, or covalently attached adducts, and other pharmacologically approved methods of delivery. Routes of administration include intramuscular, aerosol, oral (tablet or pill form),  
20 topical, systemic, ocular, intraperitoneal and/or intrathecal. Expression vectors for immunization with ribozymes and/or delivery of ribozymes are also suitable.

The specific delivery route of any selected ribozyme will depend on the use of the ribozyme. Generally, a  
25 specific delivery program for each ribozyme will focus on unmodified ribozyme uptake with regard to intracellular localization, followed by demonstration of efficacy. Alternatively, delivery to these same cells in an organ or tissue of an animal can be pursued. Uptake studies will  
30 include uptake assays to evaluate cellular ribozyme uptake, regardless of the delivery vehicle or strategy. Such assays will also determine the intracellular localization of the ribozyme following uptake, ultimately establishing the requirements for maintenance of steady-  
35 state concentrations within the cellular compartment containing the target sequence (nucleus and/or cytoplasm).



Efficacy and cytotoxicity can then be tested. Toxicity will not only include cell viability but also cell function.

Some methods of delivery that may be used include:

- 5 a. encapsulation in liposomes,
- b. transduction by retroviral vectors,
- c. conjugation with cholesterol,
- d. localization to nuclear compartment utilizing antigen binding or nuclear targeting site found
- 10 on most snRNAs or nuclear proteins,
- e. neutralization of charge of ribozyme by using nucleotide derivatives, and
- f. use of blood stem cells to distribute ribozymes throughout the body.

15 At least three types of delivery strategies are useful in the present invention, including: ribozyme modifications, particle carrier drug delivery vehicles, and retroviral expression vectors. Unmodified ribozymes, like most small molecules, are taken up by cells, albeit

20 slowly. To enhance cellular uptake, the ribozyme may be modified essentially at random, in ways which reduce its charge but maintains specific functional groups. This results in a molecule which is able to diffuse across the cell membrane, thus removing the permeability barrier.

25 Modification of ribozymes to reduce charge is just one approach to enhance the cellular uptake of these larger molecules. The random approach, however, is not advisable since ribozymes are structurally and functionally more complex than small drug molecules. The structural requirements necessary to maintain ribozyme

30 catalytic activity are well understood by those in the art. These requirements are taken into consideration when designing modifications to enhance cellular delivery. The modifications are also designed to reduce susceptibility

35 to nuclease degradation. Both of these characteristics should greatly improve the efficacy of the ribozyme. Cellular uptake can be increased by several orders of

magnitude without having to alter the phosphodiester linkages necessary for ribozyme cleavage activity.

Chemical modifications of the phosphate backbone will reduce the negative charge allowing free diffusion across the membrane. This principle has been successfully demonstrated for antisense DNA technology. The similarities in chemical composition between DNA and RNA make this a feasible approach. In the body, maintenance of an external concentration will be necessary to drive the diffusion of the modified ribozyme into the cells of the tissue. Administration routes which allow the diseased tissue to be exposed to a transient high concentration of the drug, which is slowly dissipated by systemic adsorption are preferred. Intravenous administration with a drug carrier designed to increase the circulation half-life of the ribozyme can be used. The size and composition of the drug carrier restricts rapid clearance from the blood stream. The carrier, made to accumulate at the site of infection, can protect the ribozyme from degradative processes.

Drug delivery vehicles are effective for both systemic and topical administration. They can be designed to serve as a slow release reservoir, or to deliver their contents directly to the target cell. An advantage of using direct delivery drug vehicles is that multiple molecules are delivered per uptake. Such vehicles have been shown to increase the circulation half-life of drugs which would otherwise be rapidly cleared from the blood stream. Some examples of such specialized drug delivery vehicles which fall into this category are liposomes, hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres.

From this category of delivery systems, liposomes are preferred. Liposomes increase intracellular stability, increase uptake efficiency and improve biological activity.

Liposomes are hollow spherical vesicles composed of lipids arranged in a similar fashion as those lipids which make up the cell membrane. They have an internal aqueous space for entrapping water soluble compounds and range in  
5 size from 0.05 to several microns in diameter. Several studies have shown that liposomes can deliver RNA to cells and that the RNA remains biologically active.

For example, a liposome delivery vehicle originally designed as a research tool, Lipofectin, has been shown to  
10 deliver intact mRNA molecules to cells yielding production of the corresponding protein. In another study, an antibody targeted liposome delivery system containing an RNA molecule 3,500 nucleotides in length and antisense to a structural protein of HIV, inhibited virus proliferation  
15 in a sequence specific manner. Not only did the antibody target the liposomes to the infected cells, but it also triggered the internalization of the liposomes by the infected cells. Triggering the endocytosis is useful for viral inhibition. Finally, liposome delivered synthetic  
20 ribozymes have been shown to concentrate in the nucleus of H9 (an example of an HIV-sensitive cell) cells and are functional as evidenced by their intracellular cleavage of the sequence. Liposome delivery to other cell types using smaller ribozymes (less than 142 nucleotides in length)  
25 exhibit different intracellular localizations.

Liposomes offer several advantages: They are non-toxic and biodegradable in composition; they display long circulation half-lives; and recognition molecules can be readily attached to their surface for targeting to  
30 tissues. Finally, cost effective manufacture of liposome-based pharmaceuticals, either in a liquid suspension or lyophilized product, has demonstrated the viability of this technology as an acceptable drug delivery system.

Other controlled release drug delivery systems, such  
35 as nonoparticles and hydrogels may be potential delivery vehicles for a ribozyme. These carriers have been developed for chemotherapeutic agents and protein-based

pharmaceuticals, and consequently, can be adapted for ribozyme delivery.

Topical administration of ribozymes is advantageous since it allows localized concentration at the site of administration with minimal systemic adsorption. This simplifies the delivery strategy of the ribozyme to the disease site and reduces the extent of toxicological characterization. Furthermore, the amount of material to be applied is far less than that required for other administration routes. Effective delivery requires the ribozyme to diffuse into the infected cells. Chemical modification of the ribozyme to neutralize negative charge may be all that is required for penetration. However, in the event that charge neutralization is insufficient, the modified ribozyme can be co-formulated with permeability enhancers, such as Azone or oleic acid, in a liposome. The liposomes can either represent a slow release presentation vehicle in which the modified ribozyme and permeability enhancer transfer from the liposome into the infected cell, or the liposome phospholipids can participate directly with the modified ribozyme and permeability enhancer in facilitating cellular delivery. In some cases, both the ribozyme and permeability enhancer can be formulated into a suppository formulation for slow release.

Ribozymes may also be systemically administered. Systemic absorption refers to the accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include: intravenous, subcutaneous, intraperitoneal, intranasal, intrathecal and ophthalmic. Each of these administration routes expose the ribozyme to an accessible diseased tissue. Subcutaneous administration drains into a localized lymph node which proceeds through the lymphatic network into the circulation. The rate of entry into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or

other drug carrier localizes the ribozyme at the lymph node. The ribozyme can be modified to diffuse into the cell, or the liposome can directly participate in the delivery of either the unmodified or modified ribozyme to the cell. This method is particularly useful for treating AIDS using anti-HIV ribozymes of this invention.

Also preferred in AIDS therapy is the use of a liposome formulation which can deliver oligonucleotides to lymphocytes and macrophages. This oligonucleotide delivery system inhibits HIV proliferation in infected primary immune cells. Whole blood studies show that the formulation is taken up by 90% of the lymphocytes after 8 hours at 37°C. Preliminary biodistribution and pharmacokinetic studies yielded 70% of the injected dose/gm of tissue in the spleen after one hour following intravenous administration. This formulation offers an excellent delivery vehicle for anti-AIDS ribozymes for two reasons. First, T-helper lymphocytes and macrophages are the primary cells infected by the virus, and second, a subcutaneous administration delivers the ribozymes to the resident HIV-infected lymphocytes and macrophages in the lymph node. The liposomes then exit the lymphatic system, enter the circulation, and accumulate in the spleen, where the ribozyme is delivered to the resident lymphocytes and macrophages.

Intraperitoneal administration also leads to entry into the circulation, with once again, the molecular weight or size of the ribozyme-delivery vehicle complex controlling the rate of entry.

Liposomes injected intravenously show accumulation in the liver, lung and spleen. The composition and size can be adjusted so that this accumulation represents 30% to 40% of the injected dose. The remaining dose circulates in the blood stream for up to 24 hours.

The chosen method of delivery should result in cytoplasmic accumulation in the afflicted cells and molecules should have some nuclease-resistance for optimal

dosing. Nuclear delivery may be used but is less preferable. Most preferred delivery methods include liposomes (10-400 nm), hydrogels, controlled-release polymers, microinjection or electroporation (for ex vivo  
5 treatments) and other pharmaceutically applicable vehicles. The dosage will depend upon the disease indication and the route of administration but should be between 100-200 mg/kg of body weight/day. The duration of  
10 treatment will extend through the course of the disease symptoms, usually at least 14-16 days and possibly continuously. Multiple daily doses are anticipated for topical applications, ocular applications and vaginal applications. The number of doses will depend upon  
15 disease delivery vehicle and efficacy data from clinical trials.

Establishment of therapeutic levels of ribozyme within the cell is dependent upon the rate of uptake and degradation. Decreasing the degree of degradation will prolong the intracellular half-life of the ribozyme.  
20 Thus, chemically modified ribozymes, e.g., with modification of the phosphate backbone, or capping of the 5' and 3' ends of the ribozyme with nucleotide analogues may require different dosaging. Descriptions of useful systems are provided in the art cited above, all of which  
25 is hereby incorporated by reference herein.

The claimed ribozymes are also useful as diagnostic tools to specifically or non-specifically detect the presence of a target RNA in a sample. That is, the target RNA, if present in the sample, will be specifically  
30 cleaved by the ribozyme, and thus can be readily and specifically detected as smaller RNA species. The presence of such smaller RNA species is indicative of the presence of the target RNA in the sample.

Other embodiments are within the following claims.

## "Sequence Listing"

## (1) GENERAL INFORMATION:

(i) APPLICANT: Kenneth G. Draper  
Bharat Chowrira  
5 James McSwiggen  
Daniel Stinchcomb  
James Thompson

(ii) TITLE OF INVENTION: METHOD AND REAGENT FOR  
10 INHIBITING HUMAN  
IMMUNODEFICIENCY VIRUS  
REPLICATION

(iii) NUMBER OF SEQUENCES: 68

(iv) CORRESPONDENCE ADDRESS:

15 (A) ADDRESSEE: Lyon & Lyon  
(B) STREET: 611 West Sixth Street  
(C) CITY: Los Angeles  
(D) STATE: California  
(E) COUNTRY: USA  
(F) ZIP: 90017

20 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb  
storage

(B) COMPUTER: IBM Compatible

25 (C) OPERATING SYSTEM: IBM MS-DOS (Version  
5.0)

(D) SOFTWARE: WordPerfect (Version  
5.1)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

30 (B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

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35 described below: two

(A) APPLICATION NUMBER: 08/103,243

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(A) APPLICATION NUMBER: 07/882,886  
(B) FILING DATE: May 14, 1992  
(viii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: Warburg, Richard J.  
5 (B) REGISTRATION NUMBER: 32,327  
(C) REFERENCE/DOCKET NUMBER: 206/116  
(ix) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: (213) 489-1600  
(B) TELEFAX: (213) 955-0440  
10 (C) TELEX: 67-3510  
(2) INFORMATION FOR SEQ ID NO: 1:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 40  
(B) TYPE: nucleic acid  
15 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 1:  
AGAAGAAAAG CAAAGAUCAU UAGGGAUUAU GGAAAACAGA 40  
(2) INFORMATION FOR SEQ ID NO: 2:  
20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 15  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
25 (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 2:  
AGUUUAGUAA AACAC 15  
(2) INFORMATION FOR SEQ ID NO: 3:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 15  
30 (B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 3:  
CCAUAUGUAU AUUUC 15  
35 (2) INFORMATION FOR SEQ ID NO: 4:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 15



55

(B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 4:  
 5 UCAGAAGUAC ACAUC 15

(2) INFORMATION FOR SEQ ID NO: 5:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 15  
 (B) TYPE: nucleic acid  
 10 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 5:  
 AGAUUGGUAG UAANA 15

(2) INFORMATION FOR SEQ ID NO: 6:  
 15 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 16  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 6:  
 20 AAUAACAACA UAUUGG 16

(2) INFORMATION FOR SEQ ID NO: 7:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 15  
 25 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 7:  
 AUUGGGGUCU GCAUA 15

30 (2) INFORMATION FOR SEQ ID NO: 8:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 18  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 35 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 8:  
 AUACAGGAGA AAGAGACUGG CAUUGGG 18

## (2) INFORMATION FOR SEQ ID NO: 9:

## (i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 23  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

AUCUGGGUCA GGGAGUCUCC AUA 23

## (2) INFORMATION FOR SEQ ID NO: 10:

## 10 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## 15 (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AAAAAGAGAU AUAGCACACA AGUAGACCCU 30

## (2) INFORMATION FOR SEQ ID NO: 11:

## (i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 42  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

UGAAUAUCAA GCAGGACAUACAAGGUAGG AUCUCUACAA UA 42

## 25 (2) INFORMATION FOR SEQ ID NO: 12:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
30 (D) TOPOLOGY: linear

## (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

AUACUUGGCA CUAGCAGCAU UAAUAACACC AAAAAAGAU AAGC 44

## (2) INFORMATION FOR SEQ ID NO: 13:

## (i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 19  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single

57

(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 13:  
 CACAAUGAAU GGACACUAG 19

(2) INFORMATION FOR SEQ ID NO: 14:

5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

10 (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 14:  
 AAGCUGUUAG A 11

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

15 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 15:  
 UAGGGCAACA UAUCUAUGAA ACUUA 25

20 (2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

25 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 16:  
 GCCAUAAUAA GAA 13

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 11

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 17:  
 35 AUAGGCGUUA C 11

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

58

	(A) LENGTH:	11
	(B) TYPE:	nucleic acid
	(C) STRANDEDNESS:	single
	(D) TOPOLOGY:	linear
5	(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 18:	
	GAAAUGGAGC C	11
	(2) INFORMATION FOR SEQ ID NO: 19:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH:	15
10	(B) TYPE:	nucleic acid
	(C) STRANDEDNESS:	single
	(D) TOPOLOGY:	linear
	(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 19:	
	AUCCUAGACU AGAGC	15
15	(2) INFORMATION FOR SEQ ID NO: 20:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH:	14
	(B) TYPE:	nucleic acid
	(C) STRANDEDNESS:	single
20	(D) TOPOLOGY:	linear
	(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 20:	
	AAGUCAGCCU AAAA	14
	(2) INFORMATION FOR SEQ ID NO: 21:	
	(i) SEQUENCE CHARACTERISTICS:	
25	(A) LENGTH:	26
	(B) TYPE:	nucleic acid
	(C) STRANDEDNESS:	single
	(D) TOPOLOGY:	linear
	(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 21:	
30	UGUACCAAUU GCUAUUGUAA AAAGUG	26
	(2) INFORMATION FOR SEQ ID NO: 22:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH:	12
	(B) TYPE:	nucleic acid
35	(C) STRANDEDNESS:	single
	(D) TOPOLOGY:	linear
	(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 22:	

UUCAUUGCCA AG 12

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44

5 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GUUUGUUUCA UAACAAAAGC CUUAGGCAUC UCCUAUGGCA GGAA 44

10 (2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

15 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GACAGCGACG AAGAG 15

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 14

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

25 AAGACCUCCU CAAG 14

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26

(B) TYPE: nucleic acid

30 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GGCAGUCAGA CUCAUCAAGU UUCUCU 26

(2) INFORMATION FOR SEQ ID NO: 27:

35 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11

(B) TYPE: nucleic acid

60

(C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 27:  
 AUCAAAGCAA C 11

5 (2) INFORMATION FOR SEQ ID NO: 28:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 44  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 10 (D) TOPOLOGY: linear  
 (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 28:  
 UCCCAAUCCC GAGGGGACCC GACAGGCCCG AAGGAUAGA AGAA 44

(2) INFORMATION FOR SEQ ID NO: 29:  
 (i) SEQUENCE CHARACTERISTICS:  
 15 (A) LENGTH: 15  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 29:  
 20 CAUUCGAUUA GUGAA 15

(2) INFORMATION FOR SEQ ID NO: 30:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 22  
 (B) TYPE: nucleic acid  
 25 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 30:  
 GGACGAUCUG CGGAGCCUGU GC 22

(2) INFORMATION FOR SEQ ID NO: 31:  
 30 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 29  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 35 (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 31:  
 GGGAAGCCCU CAAUAUUGG UGGAUCUC 29

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

	(A) LENGTH:	12
	(B) TYPE:	nucleic acid
	(C) STRANDEDNESS:	single
5	(D) TOPOLOGY:	linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 32:  
AGAAUAGUGC UG 12

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

10	(A) LENGTH:	16
	(B) TYPE:	nucleic acid
	(C) STRANDEDNESS:	single
	(D) TOPOLOGY:	linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 33:  
15 UGCCACAGCU AUAGCA 16

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

	(A) LENGTH:	23
	(B) TYPE:	nucleic acid
20	(C) STRANDEDNESS:	single
	(D) TOPOLOGY:	linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 34:  
AAGUAGUACA AGAAGCUUAU AGA 23

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

25	(A) LENGTH:	34
	(B) TYPE:	nucleic acid
	(C) STRANDEDNESS:	single
	(D) TOPOLOGY:	linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 35:  
30 UACCUAGAAG AAUAAGACAG GGCUUGGAAA GGAU 34

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:

	(A) LENGTH:	14
35	(B) TYPE:	nucleic acid
	(C) STRANDEDNESS:	single
	(D) TOPOLOGY:	linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 36:  
UGGUCAAAAA GUAG 14

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 22  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 37:  
10 AAGAAUGAGA CGAGCUGAGC CA 22

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 15  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 38:  
GGAGCAGUAU CUCGA 15

(2) INFORMATION FOR SEQ ID NO: 39:

20 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 39:  
25 AGACCUAGAA AAACAUGGAG CAAUCACA 28

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 34  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 40:  
CCUGGCUAGA AGCACAAGAG GAGGAGAAGG UGGG 34

35 (2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36



63

(B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 41:  
 5 ACACCUCAGG UACCUUUAAG ACCAUGACU UACAAG 36

(2) INFORMATION FOR SEQ ID NO: 42:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 40  
 (B) TYPE: nucleic acid  
 10 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 42:  
 GCAGCUGUAG AUCUUAGCCA CUUUUAAAA GAAAAGGGGG 40

(2) INFORMATION FOR SEQ ID NO: 43:  
 15 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 15  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 43:  
 20 GGGGGACUGG AAGGG 15

(2) INFORMATION FOR SEQ ID NO: 44:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 19  
 25 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 44:  
 GCUAAUUCAC UCCCAACGA 19

30 (2) INFORMATION FOR SEQ ID NO: 45:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 34  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 35 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 45:  
 AGACAAGUA UCCUUGAUCU GUGGAUCUAC CACA 34

## (2) INFORMATION FOR SEQ ID NO: 46:

## (i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 24  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

AUUGGCAGAA CUACACACCA GGAC 24

## (2) INFORMATION FOR SEQ ID NO: 47:

## 10 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## 15 (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

UCAGAUAUCC A 11

## (2) INFORMATION FOR SEQ ID NO: 48:

## (i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 15  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

AAGCUAGUAC CAGUU 15

## 25 (2) INFORMATION FOR SEQ ID NO: 49:

## (i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 14  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

GAGAACACCA GCUU 14

## (2) INFORMATION FOR SEQ ID NO: 50:

## (i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 29  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single

65

(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 50:  
 ACCCUGUGAG CCUGCAUGGA AUGGAUGAC 29

(2) INFORMATION FOR SEQ ID NO: 51:

5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

10 (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 51:  
 AGUGGAGGUU UGACAGCCGC 20

(2) INFORMATION FOR SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42

15 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 52:  
 AGUACUCAA GAACUGCUGA UAUCGAGCUU GCUACAAGGG AC 42

20 (2) INFORMATION FOR SEQ ID NO: 53:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

25 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 53:  
 CUGCUUUUUG CCUGUAC 17

(2) INFORMATION FOR SEQ ID NO: 54:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 17

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 54:  
 35 UCUGAGCCUG GGAGCUC 17

(2) INFORMATION FOR SEQ ID NO: 55:

(i) SEQUENCE CHARACTERISTICS:

66

	(A) LENGTH:	11
	(B) TYPE:	nucleic acid
	(C) STRANDEDNESS:	single
	(D) TOPOLOGY:	linear
5	(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 55:	
	UAAAGCUUGC C	11
	(2) INFORMATION FOR SEQ ID NO: 56:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH:	18
10	(B) TYPE:	nucleic acid
	(C) STRANDEDNESS:	single
	(D) TOPOLOGY:	linear
	(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 56:	
	UGCCUGUAGA UCCUAGAC	18
15	(2) INFORMATION FOR SEQ ID NO: 57:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH:	19
	(B) TYPE:	nucleic acid
	(C) STRANDEDNESS:	single
20	(D) TOPOLOGY:	linear
	(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 57:	
	AGCAUCCAGG AAGUCAGCC	19
	(2) INFORMATION FOR SEQ ID NO: 58:	
	(i) SEQUENCE CHARACTERISTICS:	
25	(A) LENGTH:	15
	(B) TYPE:	nucleic acid
	(C) STRANDEDNESS:	single
	(D) TOPOLOGY:	linear
	(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 58:	
30	CAAGUGGUCA AAANG	15
	(2) INFORMATION FOR SEQ ID NO: 59:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH:	15
	(B) TYPE:	nucleic acid
35	(C) STRANDEDNESS:	single
	(D) TOPOLOGY:	linear
	(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 59:	

GGAGCAGUAU CUCAA 15

(2) INFORMATION FOR SEQ ID NO: 60:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 60:

CCNCAGGUAC CUUUA 15

10 (2) INFORMATION FOR SEQ ID NO: 61:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 61:

GGGGGACUGG AUGGG 15

(2) INFORMATION FOR SEQ ID NO: 62:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 62:

25 CCAUAUGUAU GUUUC 15

(2) INFORMATION FOR SEQ ID NO: 63:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 63:

AGACUGGUAA UAANA 15

(2) INFORMATION FOR SEQ ID NO: 64:

35 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15

(B) TYPE: nucleic acid

68

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 64:  
AUCUGGGUCA GGGAG 15

5 (2) INFORMATION FOR SEQ ID NO: 65:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 15  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
10 (D) TOPOLOGY: linear  
(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 65:  
AUUUGGGUCA GGGAG 15

(2) INFORMATION FOR SEQ ID NO: 66:  
(i) SEQUENCE CHARACTERISTICS:  
15 (A) LENGTH: 15  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 66:  
20 CAGGGAGUCU CCAUA 15

(2) INFORMATION FOR SEQ ID NO: 67:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 15  
(B) TYPE: nucleic acid  
25 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 67:  
ACACAAGUAG ACCCU 15

(2) INFORMATION FOR SEQ ID NO: 68:  
30 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 15  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
35 (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 68:  
AACAAGGUAG GAUCU 15

Claims

1. An enzymatic nucleic acid molecule which cleaves RNA of an immunodeficiency virus in *nef*.
2. An enzymatic nucleic acid molecule which cleaves  
5 RNA of an immunodeficiency virus in a region selected from the group consisting of *tar*, *rre* and the 3'LTR region.
3. The enzymatic nucleic acid molecule of claim 1, wherein said RNA molecule is in a hammerhead motif.
4. The enzymatic nucleic acid molecule of claim 1,  
10 wherein said RNA molecule is in a hairpin, hepatitis Delta virus, group 1 intron, or RNaseP RNA motif.
5. An enzymatic nucleic acid molecule which cleaves the sequence shown as any of SEQ. ID. NOS. 1-68.
6. The enzymatic nucleic acid molecule of any of  
15 claims 1-5, wherein said ribozyme comprises between 5 and 23 bases complementary to the RNA of said gene or region.
7. The enzymatic nucleic acid of claim 6, wherein said ribozyme comprises between 10 and 18 bases complementary to the RNA of said gene or region.
- 20 8. A mammalian cell including an enzymatic nucleic acid molecule of any of claims 1-5.
9. The cell of claim 8, wherein said cell is a human cell.
10. The cell of claim 9, wherein said cell is a T4  
25 lymphocyte having a CD4 receptor molecule on its cell surface.

11. An expression vector comprising nucleic acid encoding the enzymatic nucleic acid molecule of any of claims 1-5, in a manner which allows expression of that enzymatic RNA molecule within a mammalian cell.

5        12. A method for treatment of an acquired immunodeficiency disease by administering to a patient an enzymatic nucleic acid molecule of any of claims 1-5.

13. The method of claim 12, wherein said patient is a human, cat or simian.

10        14. A method for providing defective viral particles comprising the step of contacting a cell infected with an immunodeficiency virus with an enzymatic nucleic acid molecule active to cleave a gene required for viral replication or infectivity.

15        15. The method of claim 14, wherein said molecule is active to cleave a gene required for viral protein synthesis.

16. The method of claim 14, wherein said gene is the nef or tat gene.

20        17. A defective viral particle produced by the method of any of claims 14, 15 or 16.

18. An immunogenic preparation comprising a defective viral particle formed by the method of any of claims 14, 15 or 16.

25        19. A method for immunization against infection by HIV-1, comprising the step of contacting a patient with a vector encoding an enzymatic nucleic acid molecule of any of claims 1-5.



20. Enzymatic nucleic acid molecule which cleaves a substrate sequence shown in Table VII or VIII.

21. Enzymatic nucleic acid molecule having a sequence shown in Table VII or VIII.

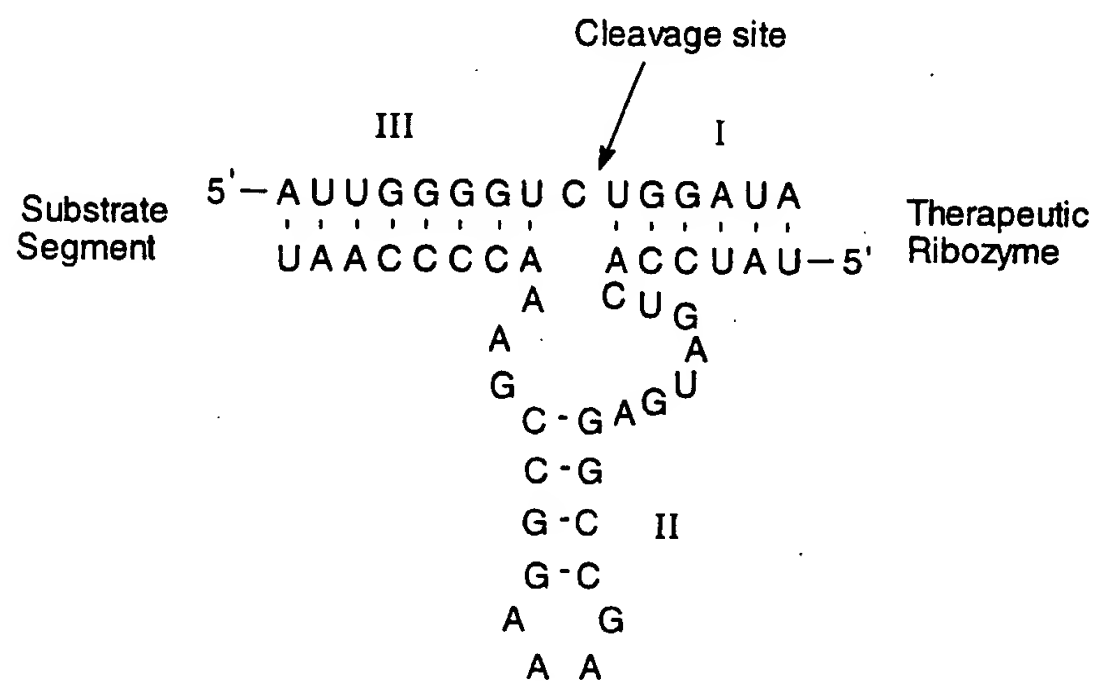
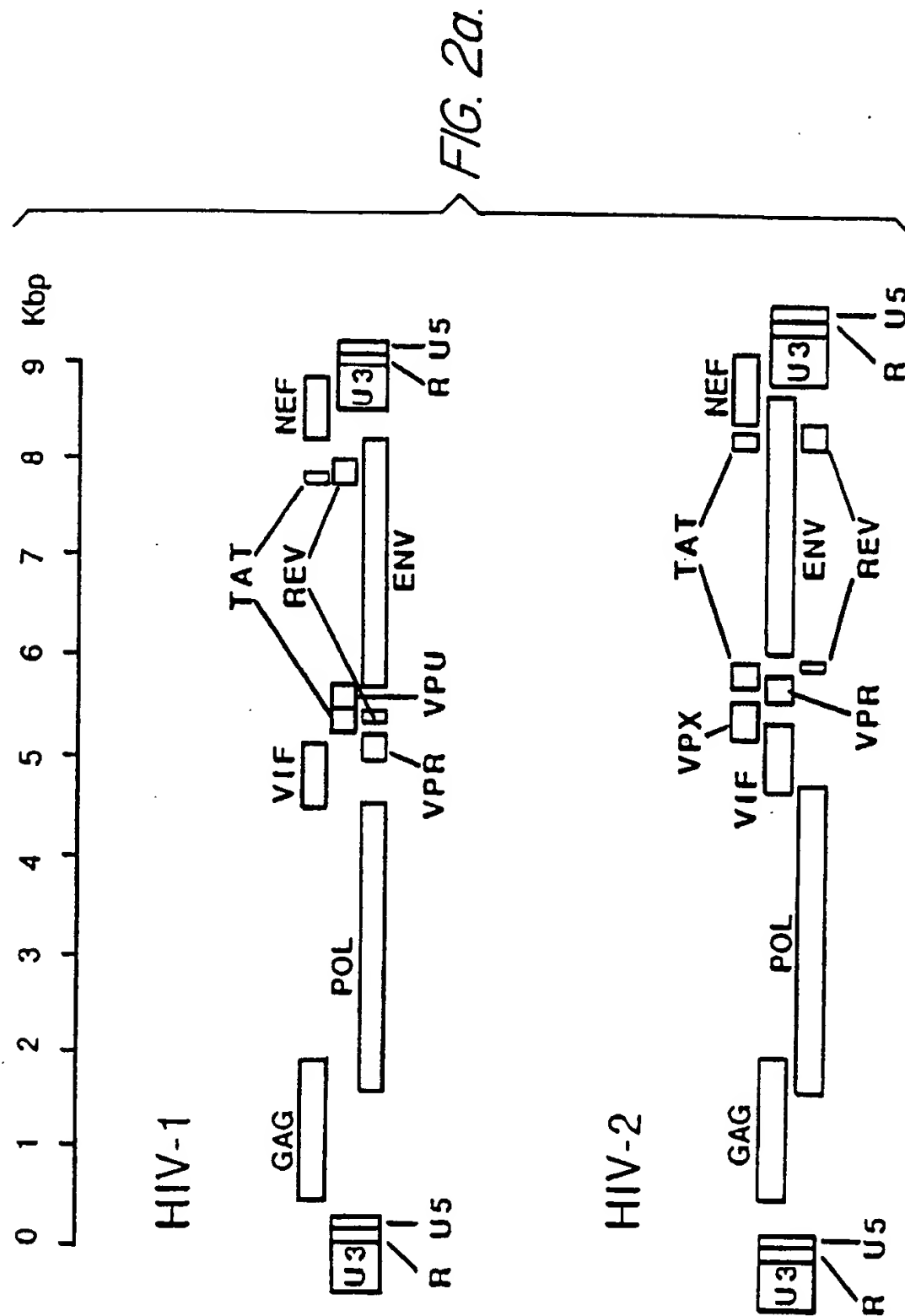


FIG. 1.



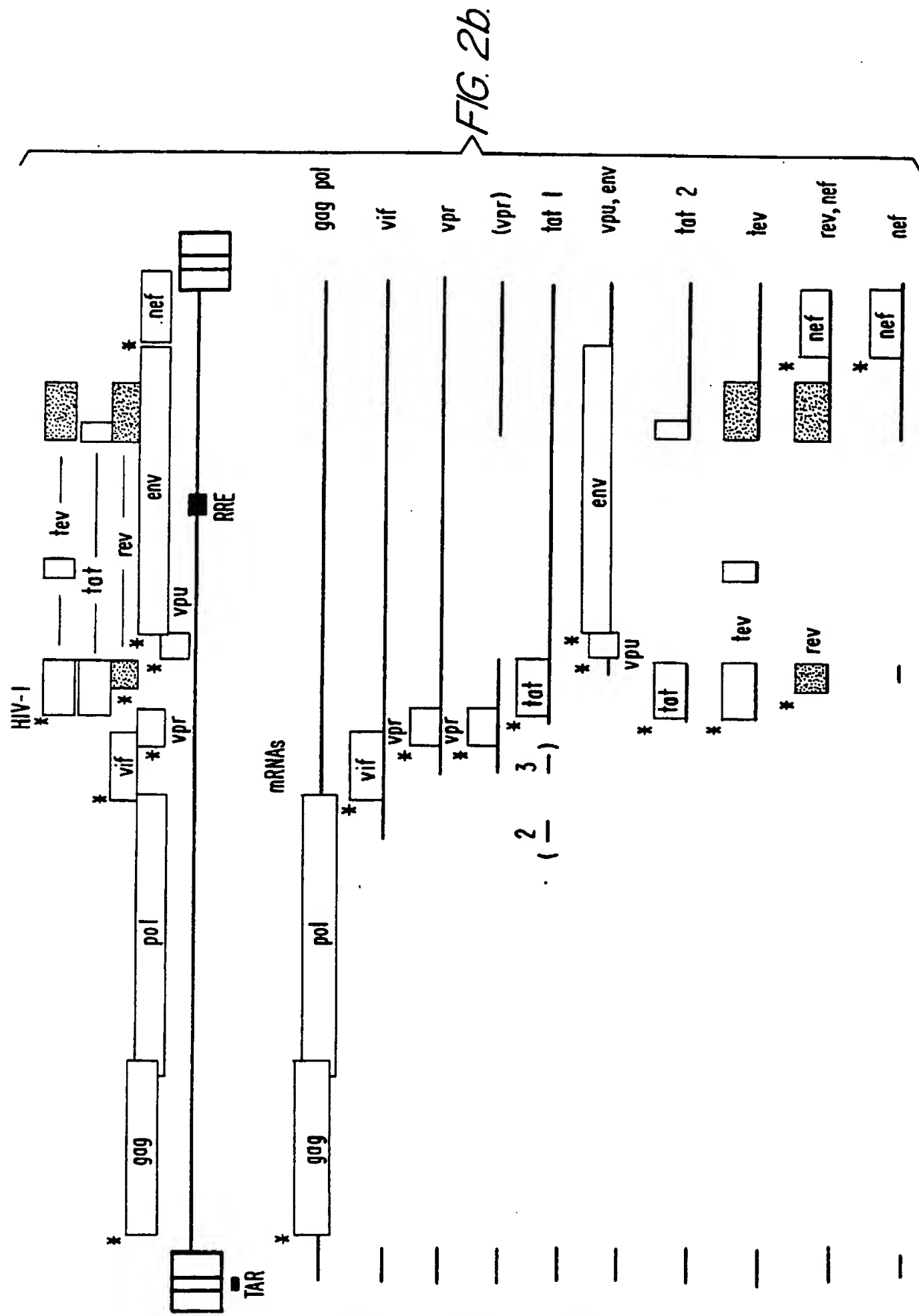


FIG. 3a.

HDH-r34MF

ENERGY = -15.5 kcal

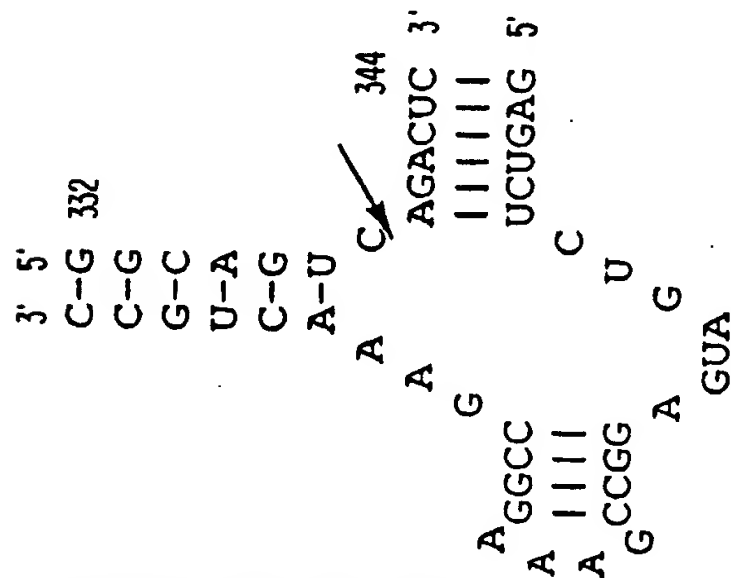


FIG. 3b.

HEH-r34MF

ENERGY = -10.7 kcal

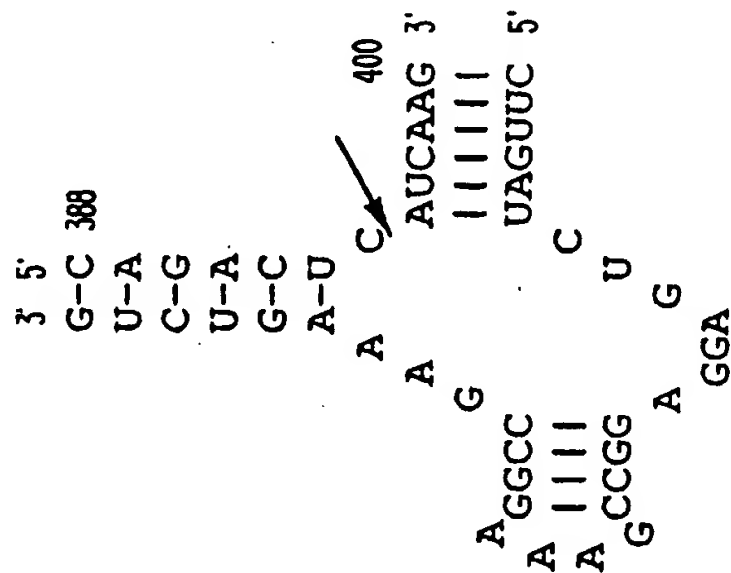
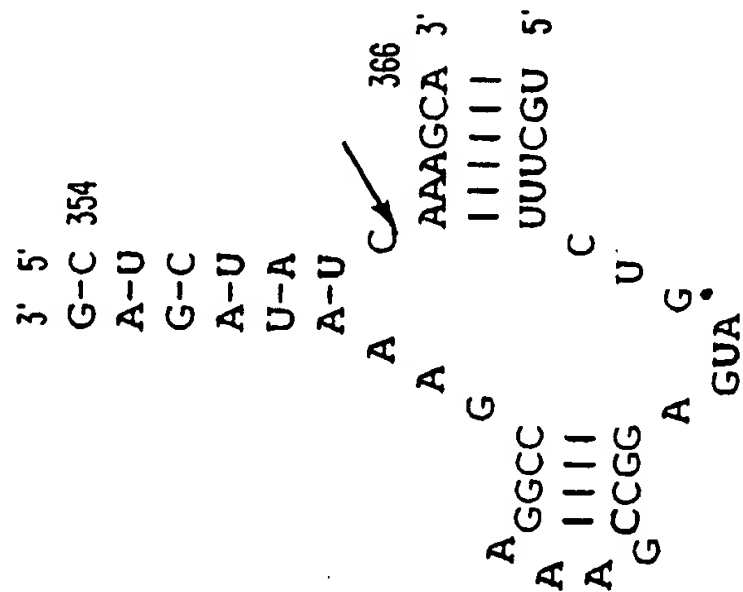


FIG. 3c.

HFH-r34MF

ENERGY = -9.9 kcal



5 / 2 3

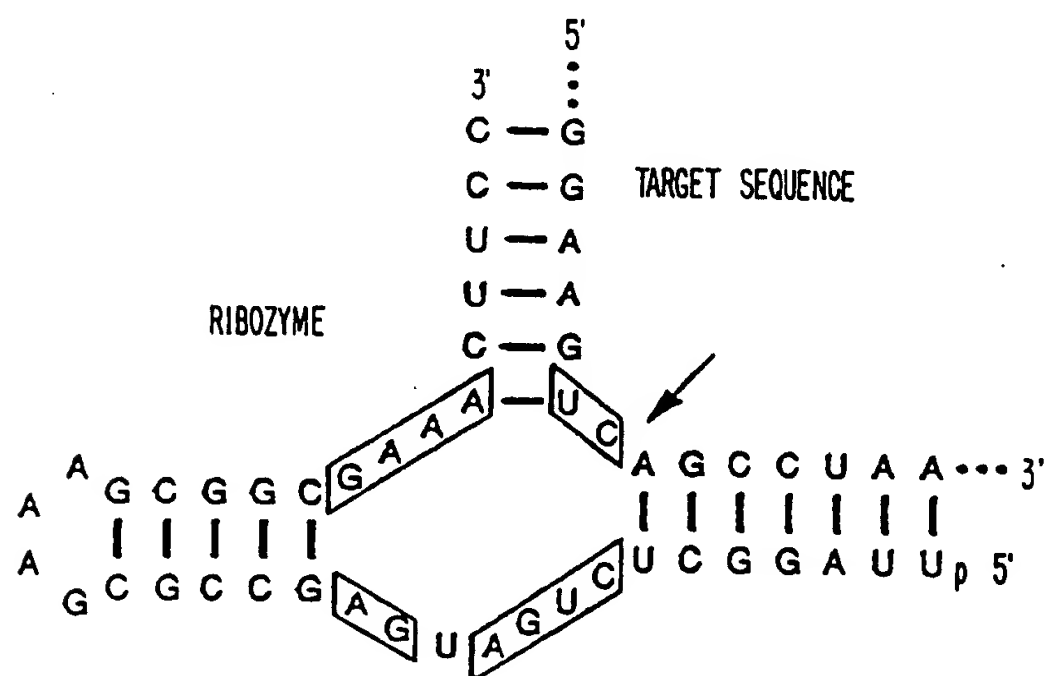


FIG. 4a.

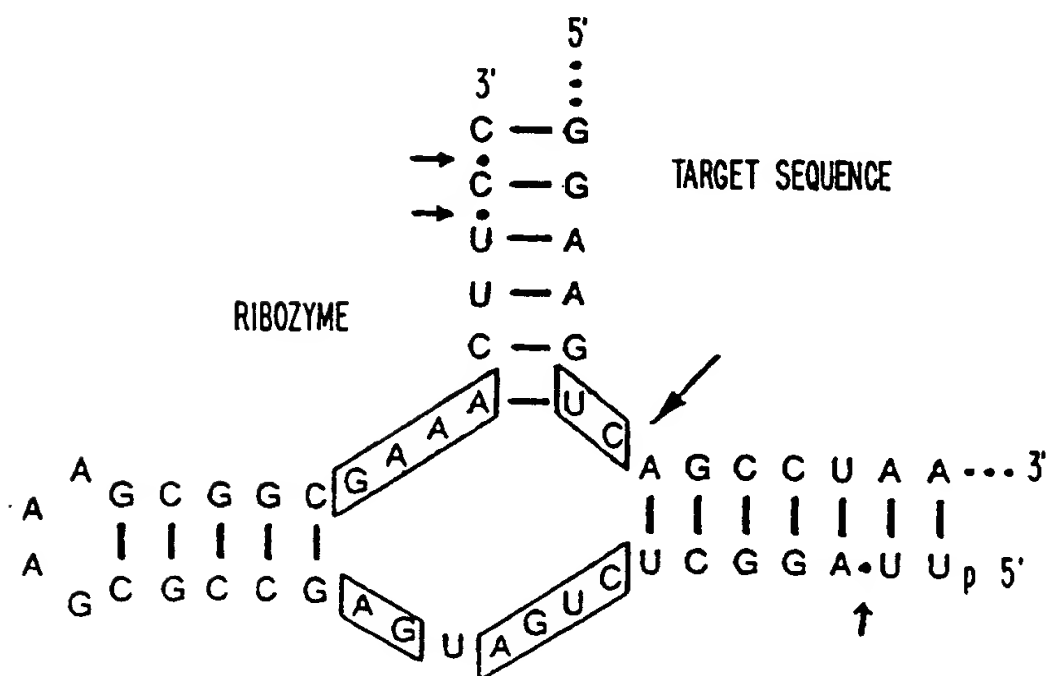


FIG. 4b.

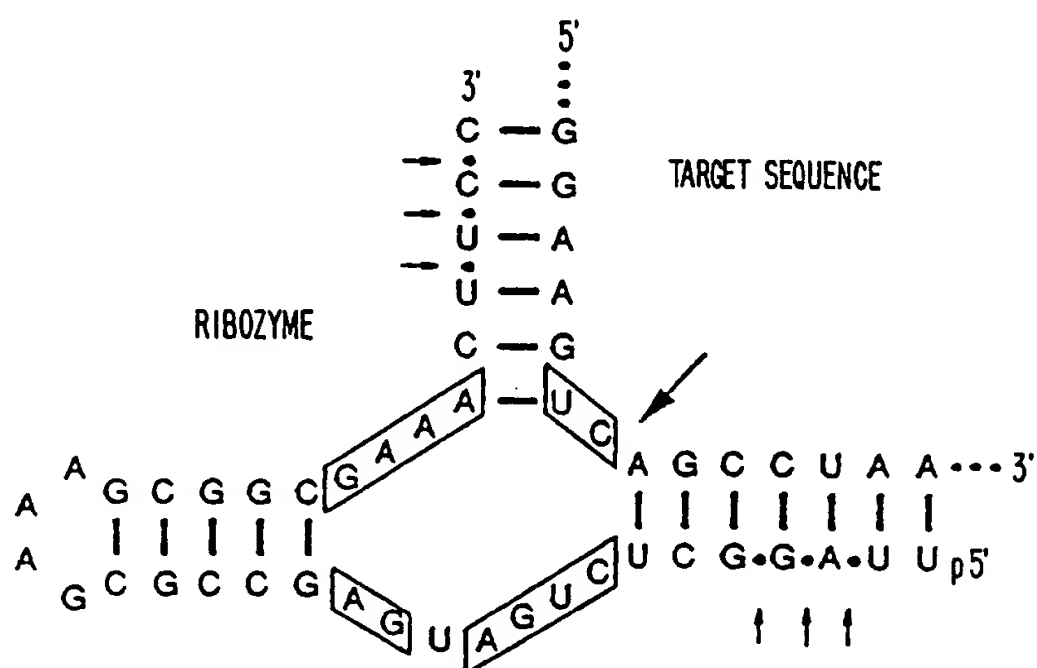
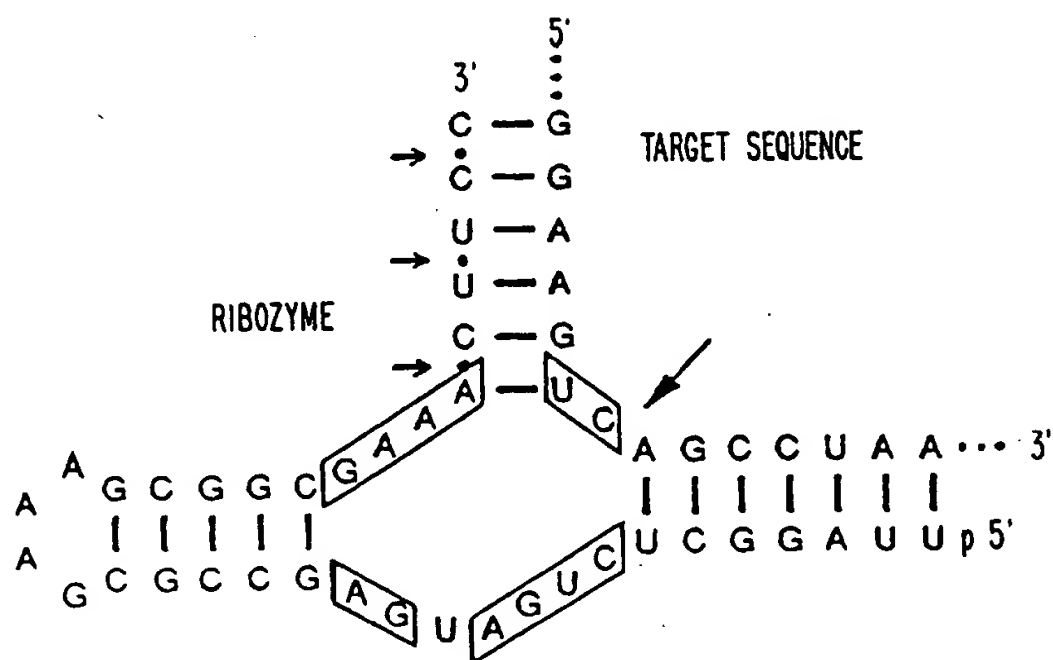
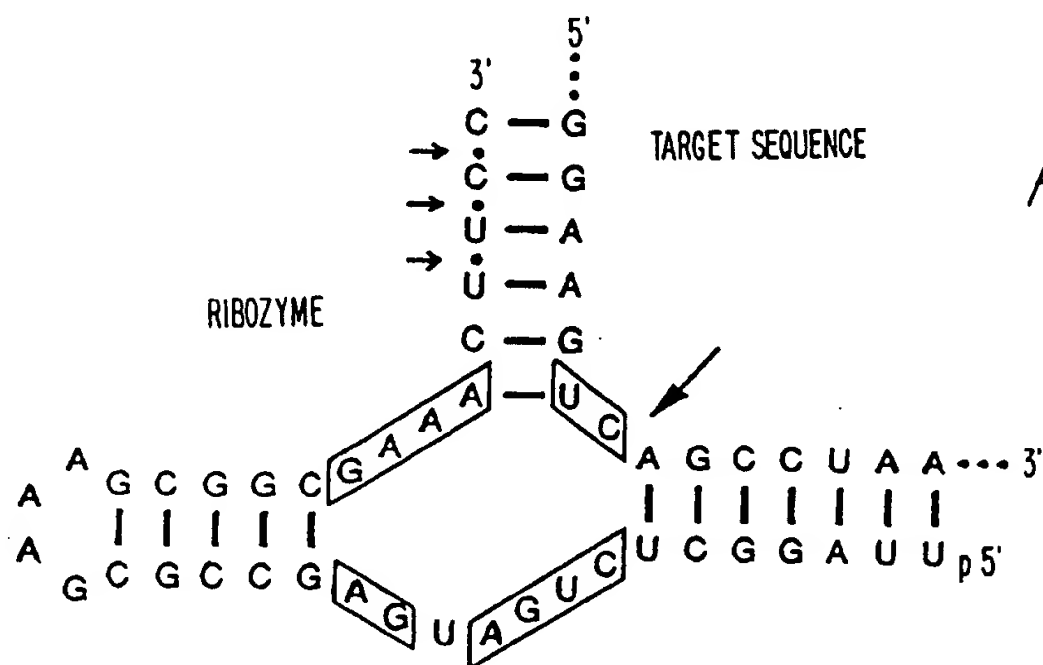


FIG. 4c.





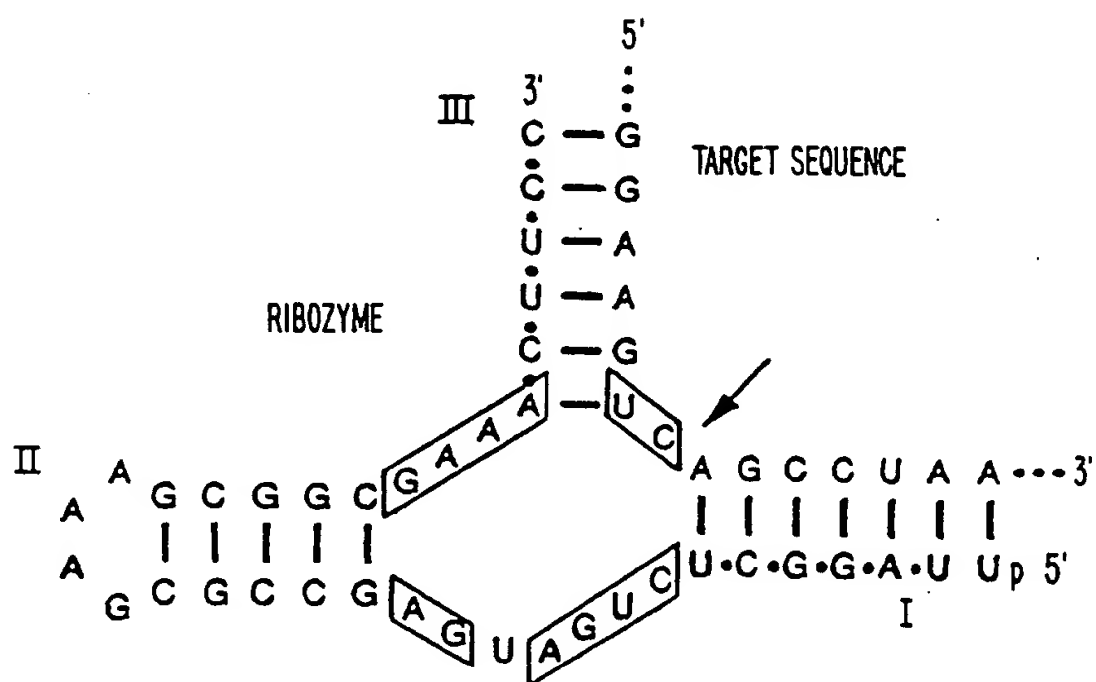


FIG. 4f.

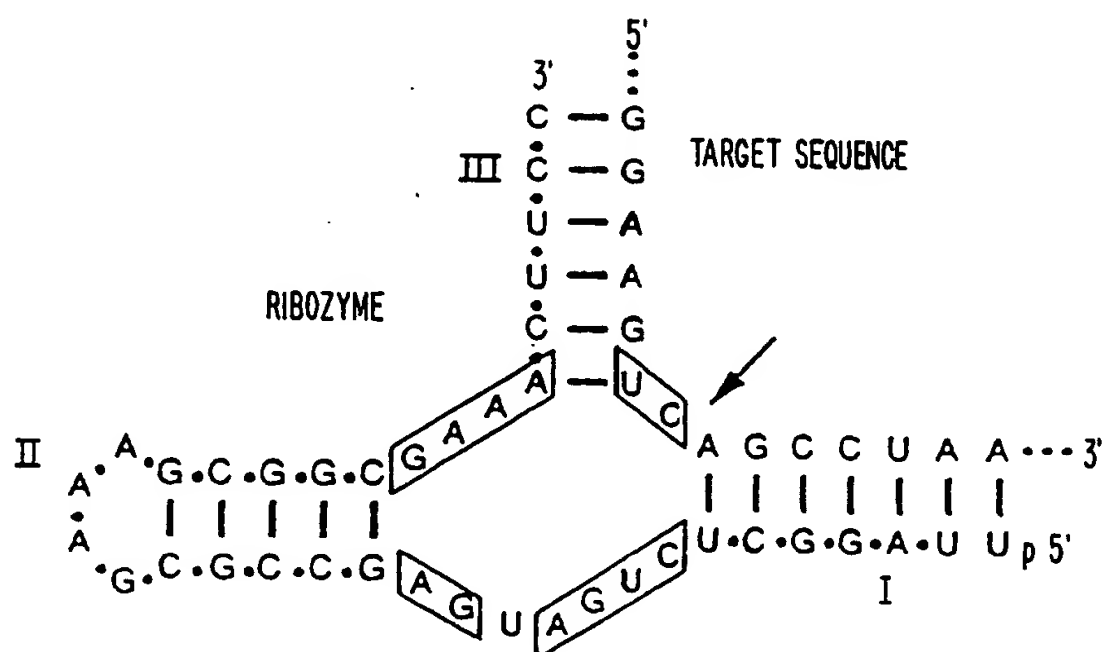


FIG. 4g.

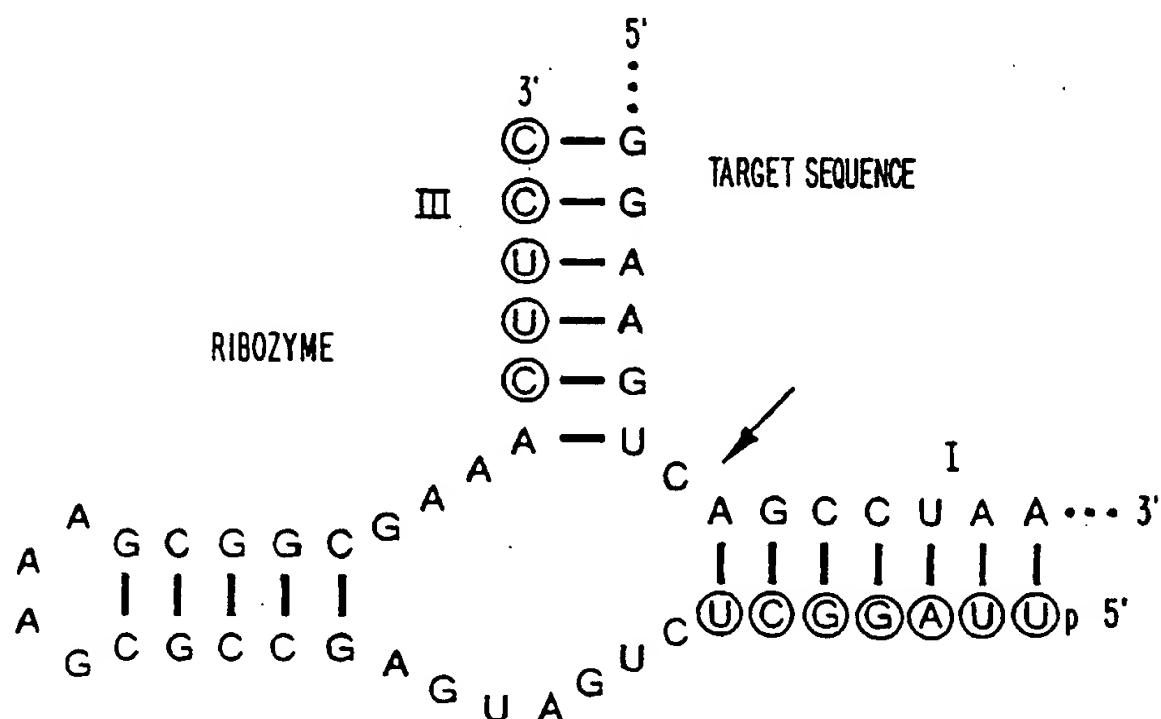


FIG. 5.

FIG. 6a.

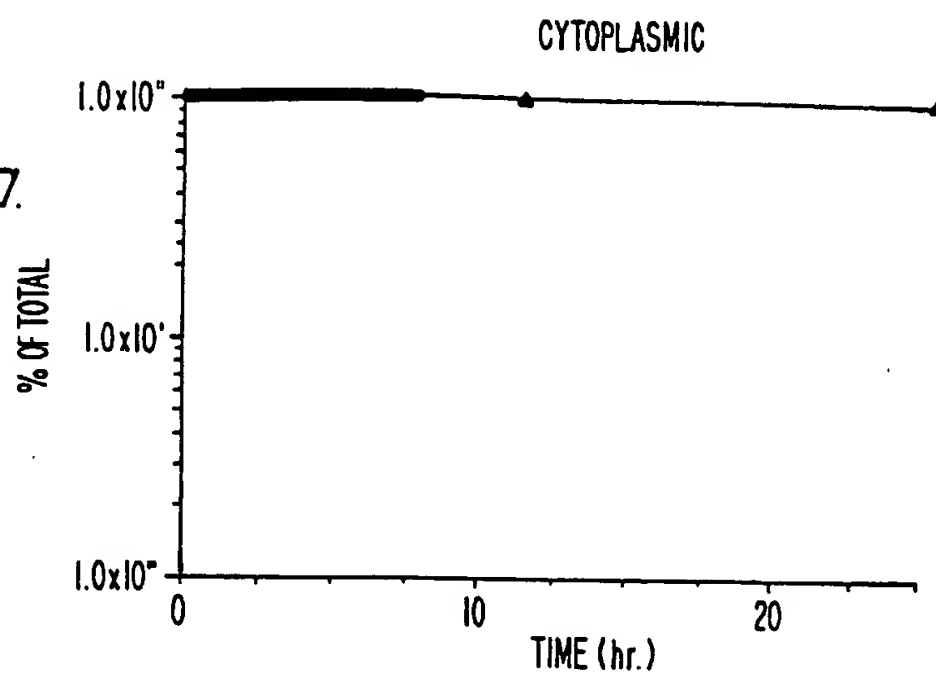


FIG. 6b.

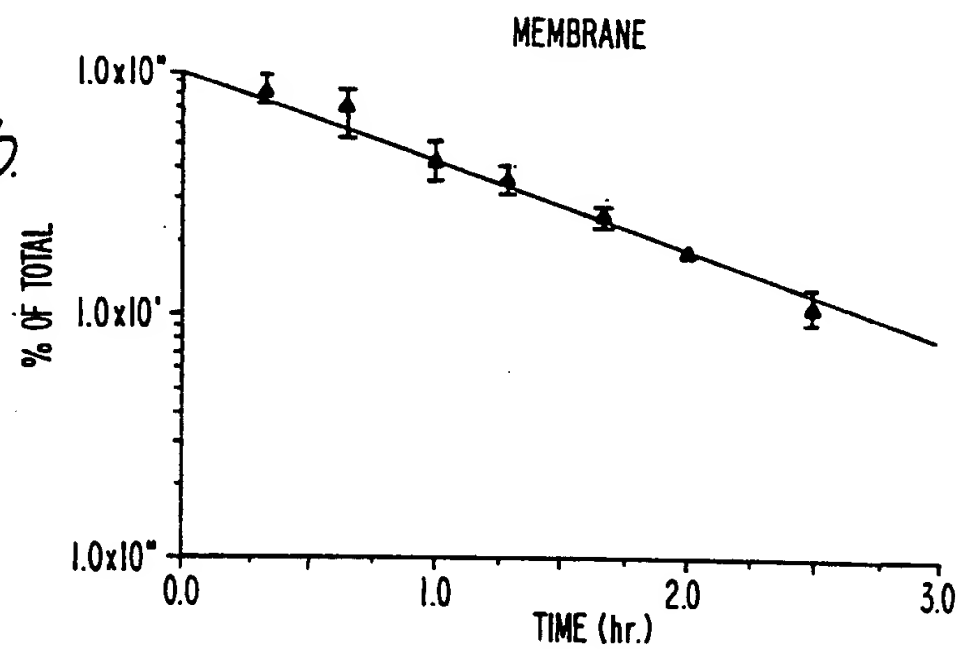


FIG. 6c.

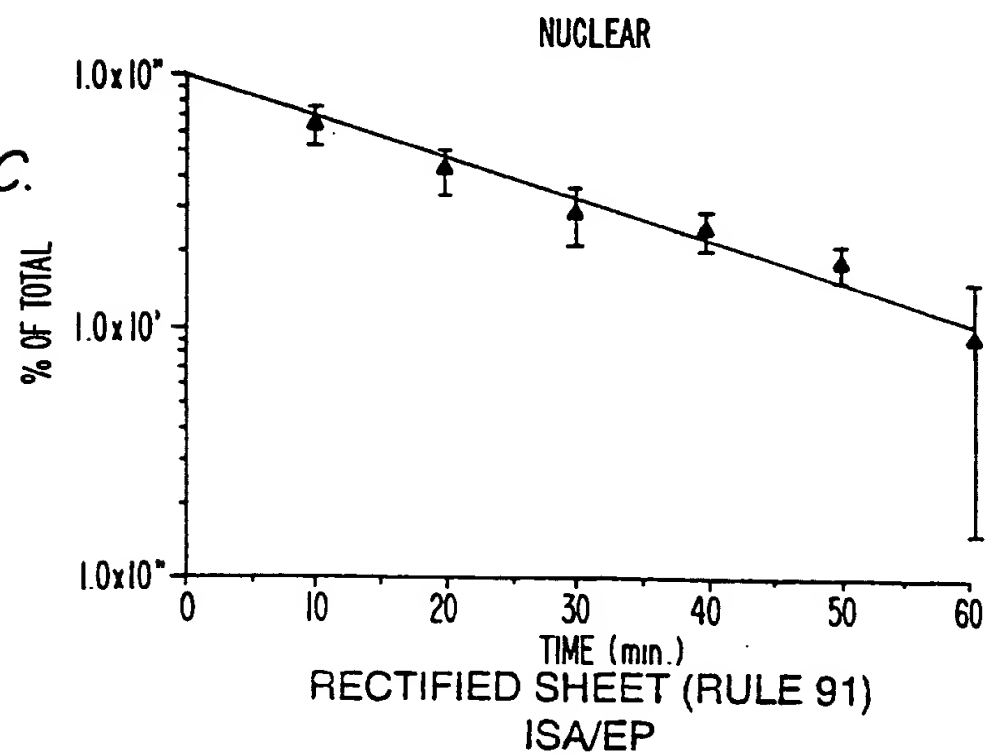


FIG. 6d.

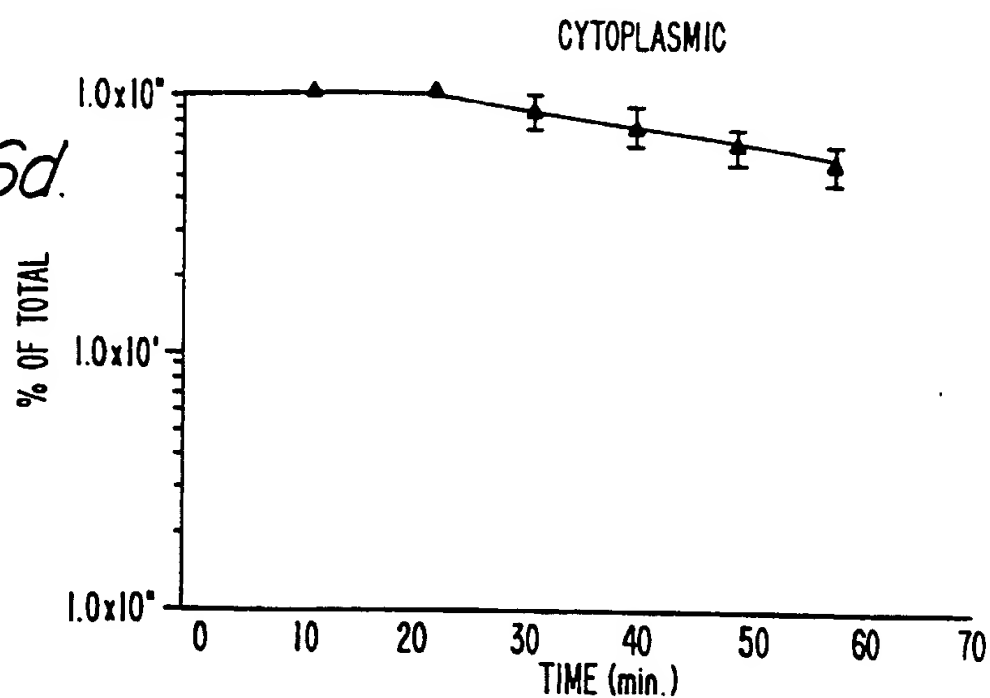


FIG. 6e.

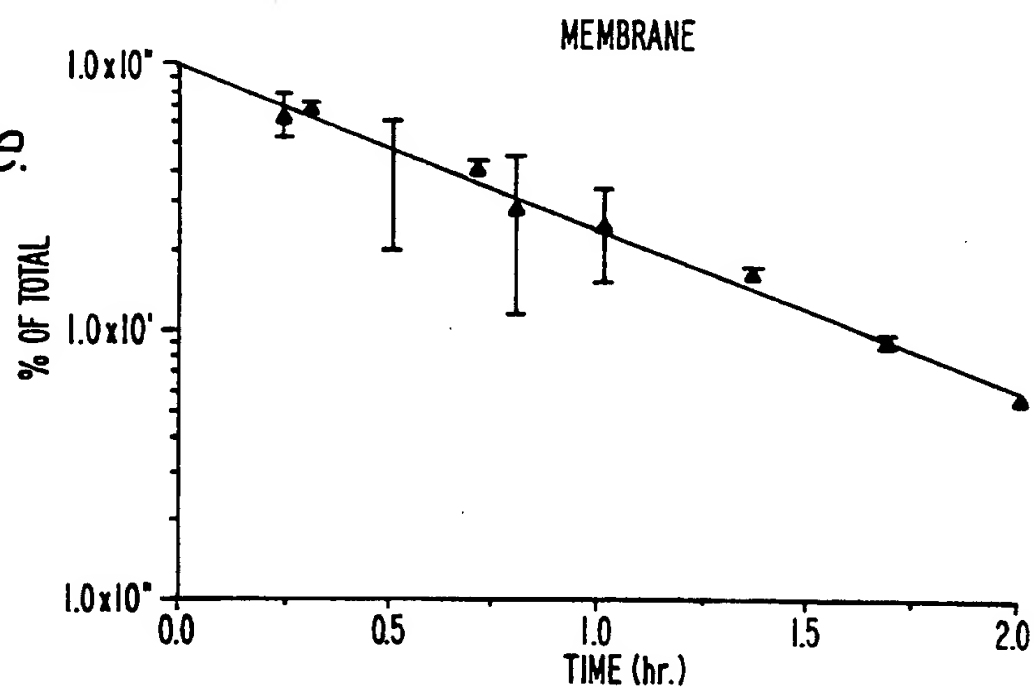
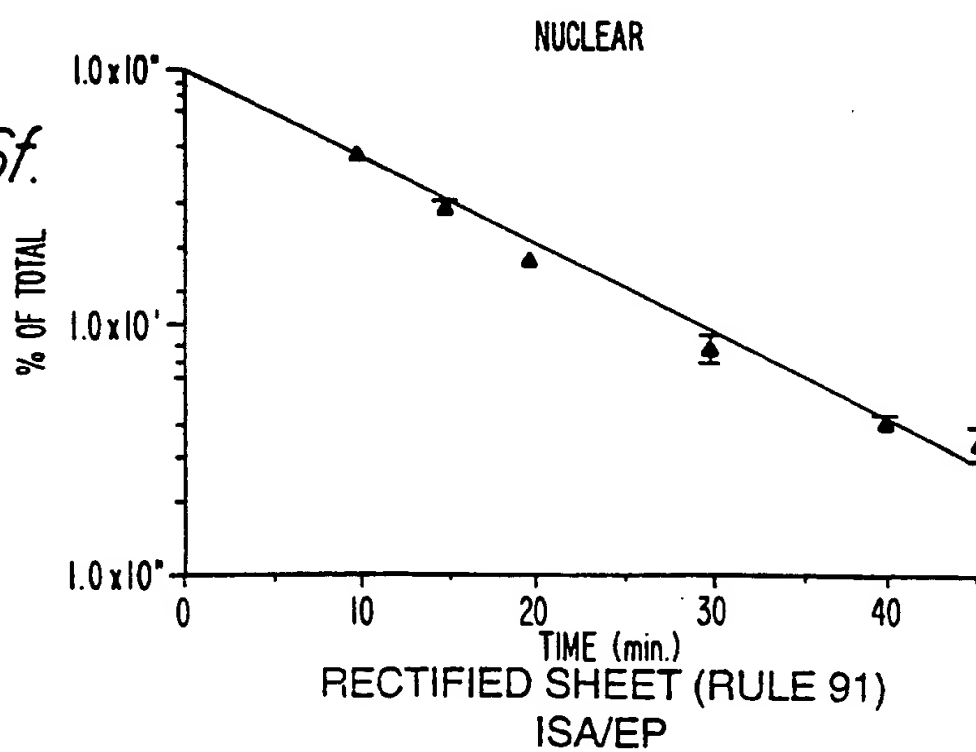


FIG. 6f.





1 3 / 2 3

FIG. 8.

RPI	3'-end	CORE	5'-end	RELATIVE RESISTANCE	
				MONO	LYMPH
1197	•	•	•	1	1
1200	O-Me	•	O-Me	2	2
1370	O-Allyl	•	O-Allyl	3	2
1414	O-Me/FU	FU7	O-Me/FU	3	1
1368	•	T7	•	3	1
1431	O-Me	NH2-U4+7	O-Me	•	2
1394	O-Me	•	O-Me	3	4 (46-mer)

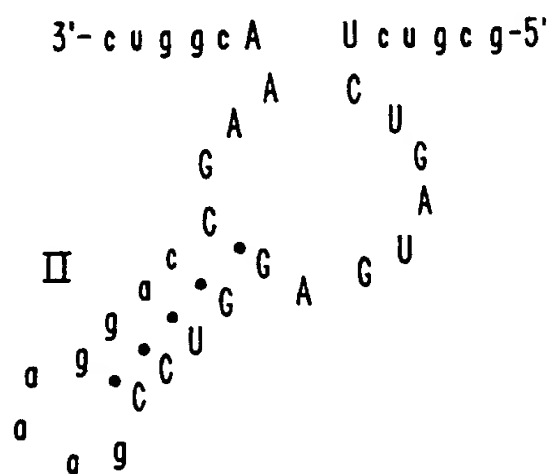


FIG. 9.

RPI	3'-end	CORE	5'-end	5'-FRAGMENT		3'-FRAGMENT	
				MONO	LYMPH	MONO	LYMPH
1197	•	•	•	2	2,9	20,23	20, 23
1200	0-Me	•	0-Me	4,14	9	20,23	20, 23
1370	0-Allyl	•	0-Allyl	2	2,9	20,23	20, 23
1414	0-Me/FU	FU7	0-Me/FU	2	9,13	20,23	20, 23
1368	•	T7	•	3	2,9	20,23	20, 23
1431	0-Me	NH2-U4+7	0-Me	3,12	9,13	20,23	20, 23
1394	0-Me	•	0-Me	2,12	9,13	8	8 (46-mer)

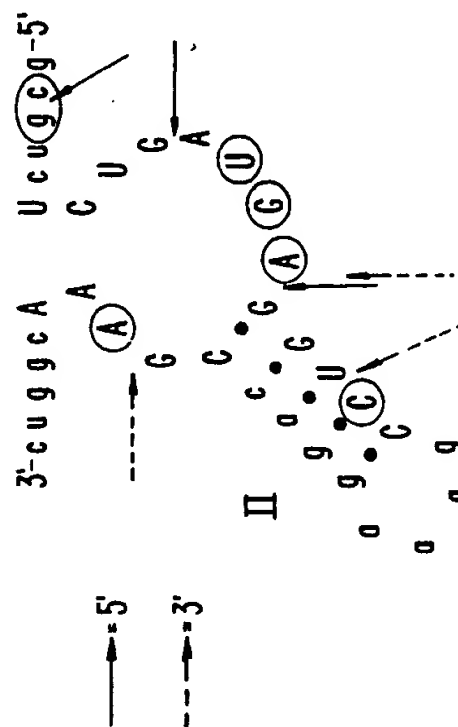
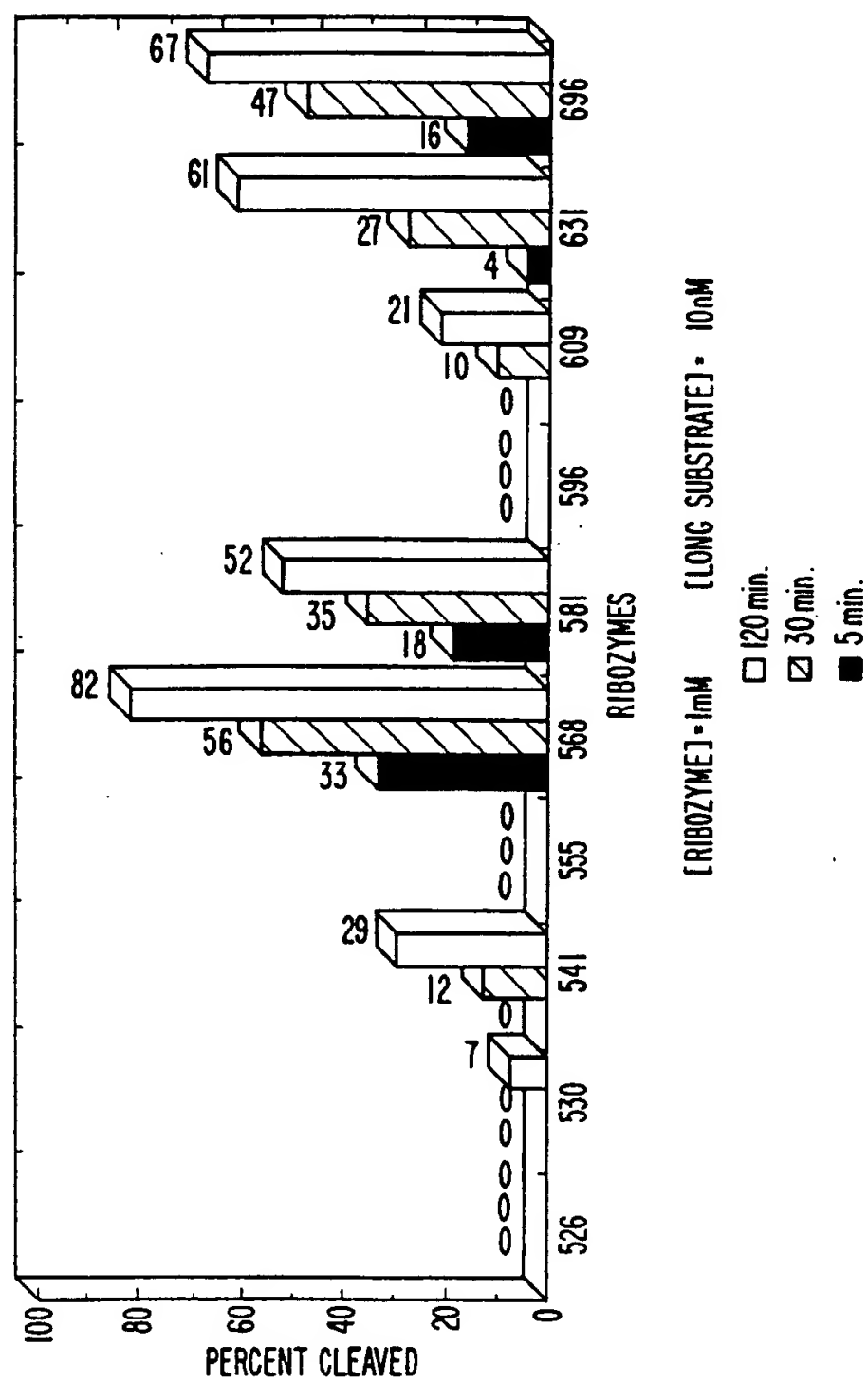


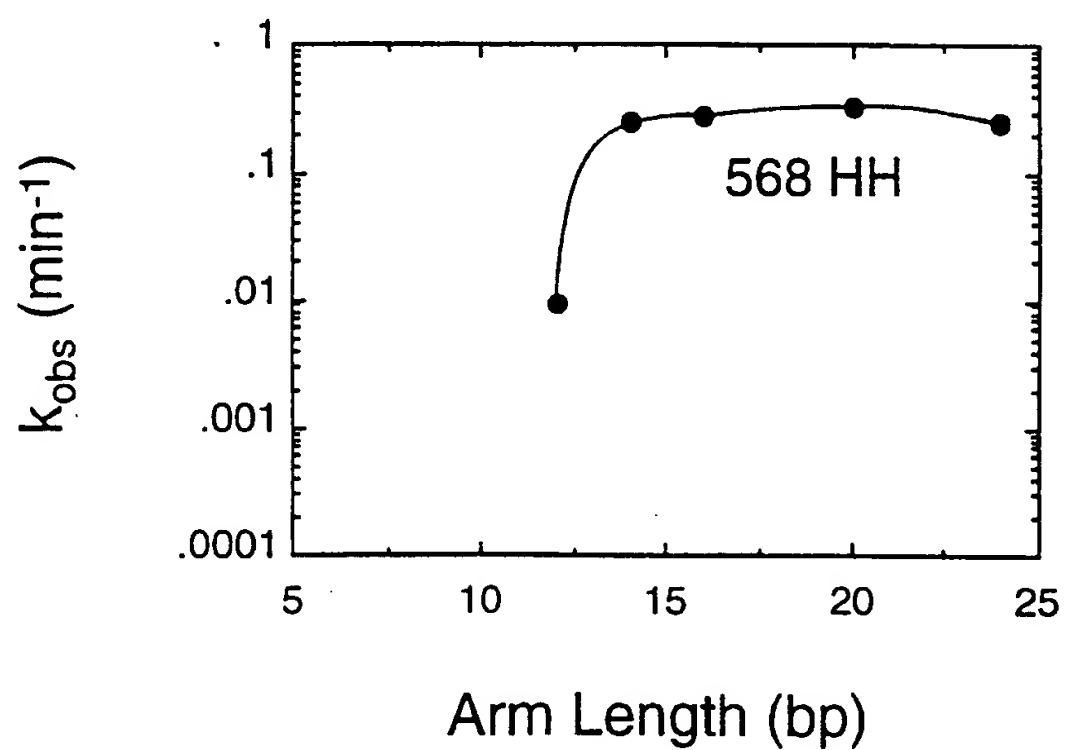
FIG. 10.





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FIG. 11.

[Ribozyme] = 1  $\mu\text{M}$ 

[Long Sub.] = &lt;10 nM

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FIG. 12.

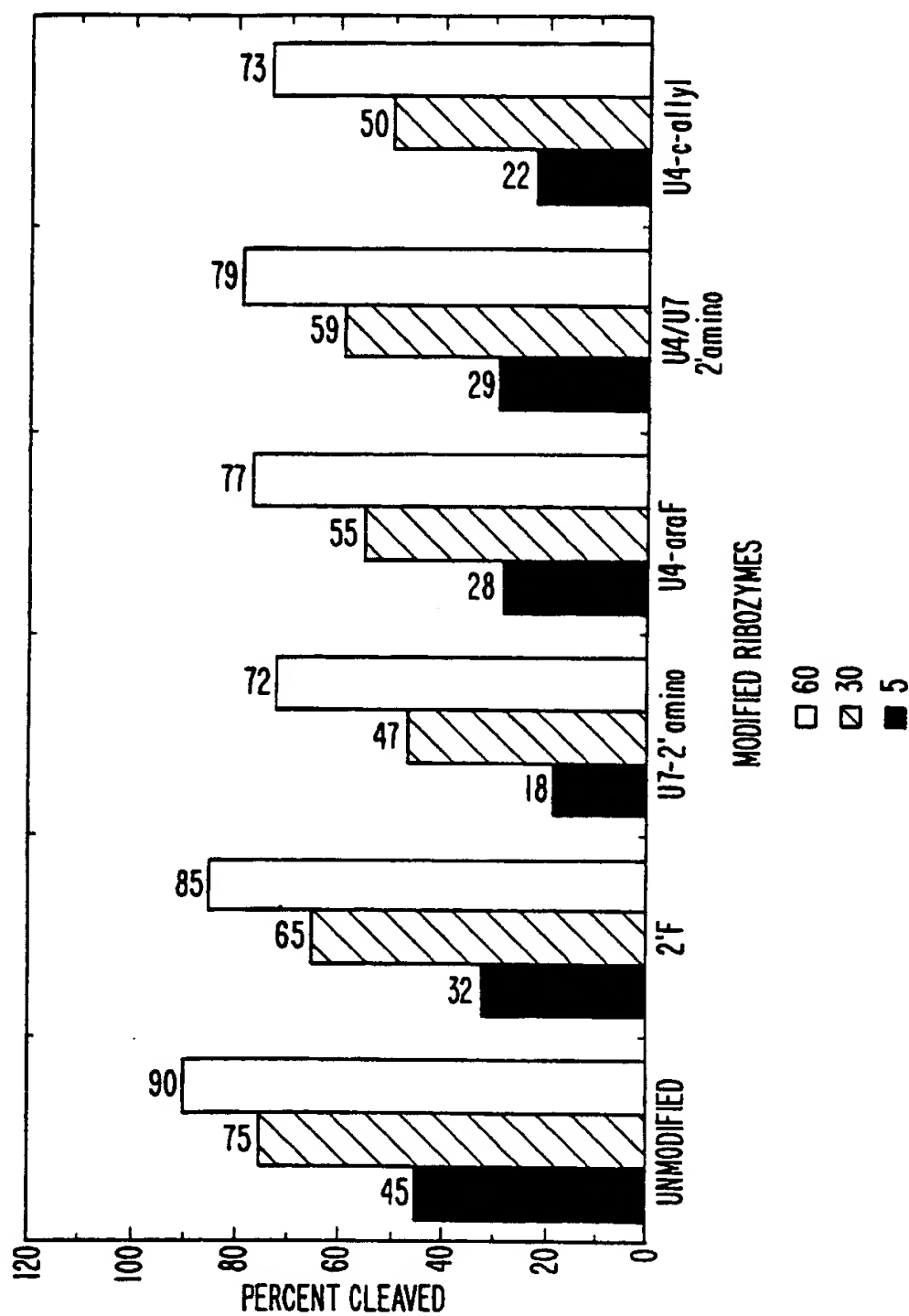


FIG. 13.

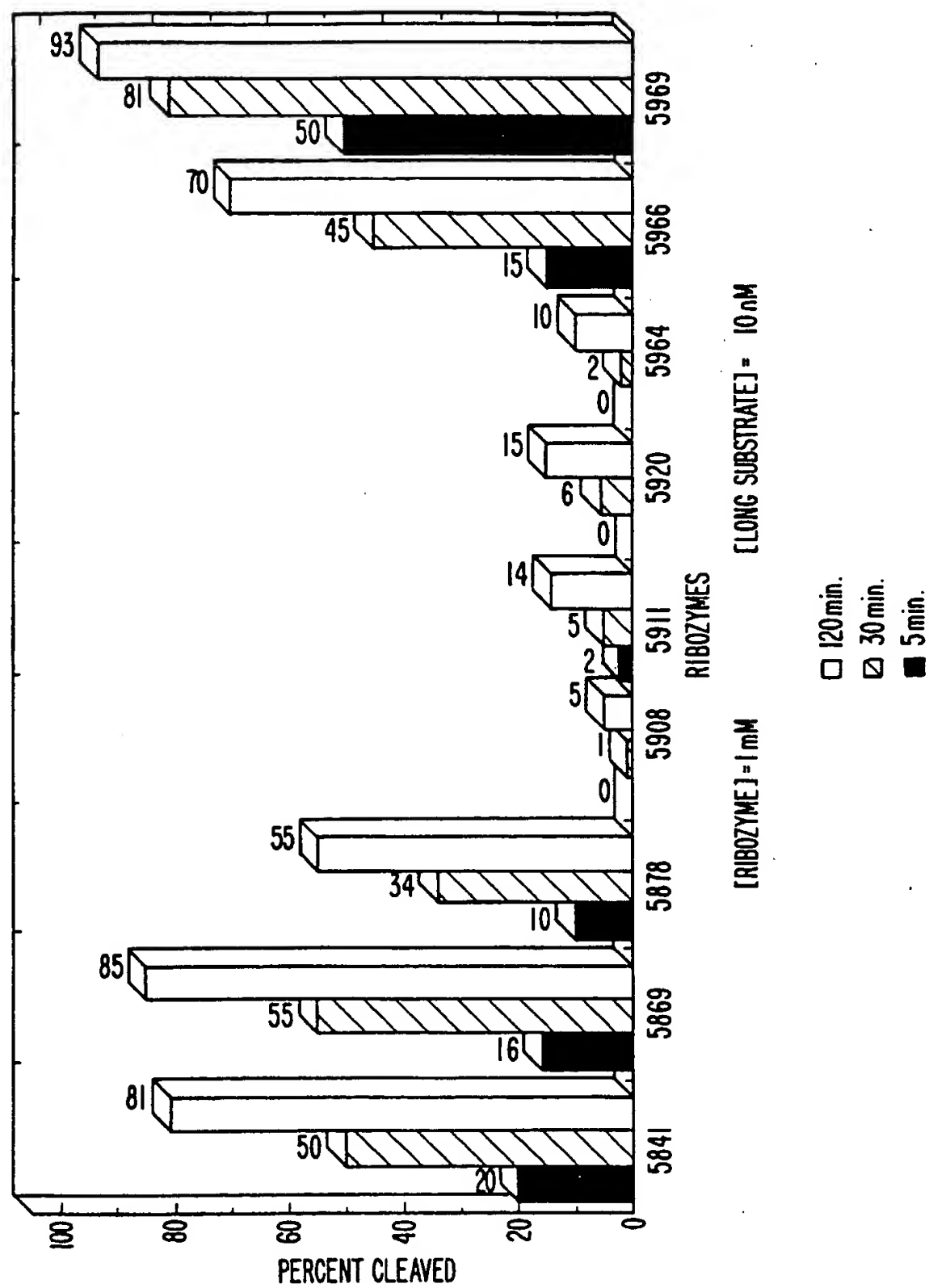


Table VII

Type	nt. Position	Hairpin Ribozyme Sequence	Substrate Sequence
HP	121	CACCAUCCAAAG AGAA GUGG ACCAGAGAAACACACGUGUGGUACAUAUACCUUGGUA	CCACU GAC CUUUGGAUGGUG
HP	515	UAUUGAGGCTUA AAGA GUGG ACCAGAGAAACACACGUGUGGUACAUAUACCUUGGUA	CCACU GCU UAAAGCCUCAUA
HP	565	AGUCACACAACA AGAA GGCA ACCAGAGAAACACACGUGUGGUACAUAUACCUUGGUA	UGCCC GUC UGUUGUGUGACU
HP	565	AGUCACACAACA AGAAGGCACAACACAGAGAAACACACGUGUGGUACAUAUACCUUGGUA	UGCCC GUC UGUUGUGUGACU
HP	2025	UCCACAUUUC AAGA GCCC ACCAGAGAAACACACGUGUGGUACAUAUACCUUGGUA	GGGU GUU GGAAUUGUGGAA
HP	4398	GCCAUUUCCTG AGAAGCAGUCACAGAGAAACACACGUGUGGUACAUAUACCUUGGUA	CUGUA GUC CAGGAUAUUGGC
HP	4398	GCCAUUUCCTG AGAA GCAG ACCAGAGAAACACACGUGUGGUACAUAUACCUUGGUA	CUGUA GUC CAGGAUAUUGGC
HP	9205	CACCAUCCAAAG AGAA GUGG ACCAGAGAAACACACGUGUGGUACAUAUACCUUGGUA	CCACU GAC CUUUGGAUGGUG
HP	9599	UAUUGAGGCTUA AAGA GUGG ACCAGAGAAACACACGUGUGGUACAUAUACCUUGGUA	CCACU GCU UAAAGCCUCAUA
HP	9649	AGUCACACAACA AGAA GGCA ACCAGAGAAACACACGUGUGGUACAUAUACCUUGGUA	UGCCC GUC UGUUGUGUGACU
Type	nt. Position	Hammerhead Ribozyme Sequence	Substrate Sequence
HH	520	UUUAUUGAGGCU CUGAUGAGGCCGAAAGGCCGAA AAGCAGUGGGUU	AACCCACUGCUUA AGCCUCAUAUA
HH	526	GCAAGCUUUAU CUGAUGAGGCCGAAAGGCCGAA AGGCUUAAGCAG	CUGCUUAAGCCUC AAUAAGCUUGC
HH	530	CAAGGCAAGCUU CUGAUGAGGCCGAAAGGCCGAA AUUGAGGCUUA	UUAAAGCCUCAUA AAGCUUGCCUUG
HH	541	CUUGAAGCACUC CUGAUGAGGCCGAAAGGCCGAA AGGCAAGCUUA	UAAAGCUUGCCUU GAGUGCUUCAAG
HH	555	ACGGGCACAC CUGAUGAGGCCGAAAGGCCGAA ACUUGAAGCACU	AGUGCUUCAAGUA GUGUGUGCCCGU
HH	568	AGUCACACAACA CUGAUGAGGCCGAAAGGCCGAA ACGGGCACAC	GUGUGUGCCCGUC UGUUGUGUGACU
HH	581	CUUAGUUACCA CUGAUGAGGCCGAAAGGCCGAA AGUCACACAACA	UGUUGUGUGACUC UGGUAACUAGAG
HH	596	AAGGUCUGAGG CUGAUGAGGCCGAAAGGCCGAA AUCUCUAGUUAC	GUAACUAAGAGAU CCUCAGACCCUU
HH	609	CCACACUGACUA CUGAUGAGGCCGAAAGGCCGAA AAGGUCUGAGG	CCUCAGACCCUUU UAGUCAGUGUGG
HH	631	GGGCGCCACUGC CUGAUGAGGCCGAAAGGCCGAA AGAGAUUUUCCA	UGGAAAUCUCUA GCAGUGGCCGCC
HH	696	CUUCAGCAAGCC CUGAUGAGGCCGAAAGGCCGAA AGUCCUGCGUGG	CGACGCAAGGACUC GGUUGGUGAAG
HH	805	CCGCUUAUAUACU CUGAUGAGGCCGAAAGGCCGAA ACGCUCUCGCAC	GUGCGAGAGCGUC AGUAUAUAGCGG
HH	2028	UCCACAUUUC CUGAUGAGGCCGAAAGGCCGAA ACAGCCUUUUU	AAAAGGGGCTGUU GGAAUUGUGGAA
HH	4398	AUAUUCCTGGAC CUGAUGAGGCCGAAAGGCCGAA ACAGUCUACUUG	CAAGUAGACUGUA GUCCAGGAUAU
HH	5841	UAGUCUAGGAUC CUGAUGAGGCCGAAAGGCCGAA ACUGGCUCCAUU	AAUGGAGCCAGUA GAUCCUAGACUA
HH	5869	GCUGACUUCCTUG CUGAUGAGGCCGAAAGGCCGAA AUGCUCUCCAGG	CCUUGGAAGCAUC CAGGAAGUCAGC
HH	5878	CAGUUUUUAGGCU CUGAUGAGGCCGAAAGGCCGAA ACUUCCTUGAUG	CAUCCAGGAAGUC AGCCUAAAACUG

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HH	5908	AACACUUUUUAC	CUGAUGAGGCCGAAAGGCCGAA	AUAGCAAUUGGU	ACCAAUUGCUAUU	GUAAAAAGUGUU
HH	5911	AGCAACACUUUU	CUGAUGAGGCCGAAAGGCCGAA	ACAAUAGCAAUU	AAUUGCUAUUGUA	AAAAGUGUUGCU
HH	5920	GGCAAUUGAAAGC	CUGAUGAGGCCGAAAGGCCGAA	ACACUUUUUACA	UGUAAAAAGUGUU	GCUUUCAUUGCC
HH	5964	CCUGCCAUAGGA	CUGAUGAGGCCGAAAGGCCGAA	AUGCCUAAAGGCU	AGCCUUAGGCAUC	UCCUAUGGCAGG
HH	5966	UUCCTUGCCAUAG	CUGAUGAGGCCGAAAGGCCGAA	AGAUGCCUAAAGG	CCUUAGGCAUCUC	CUAUGGCAGGAA
HH	5969	UUCUUCCUGCCA	CUGAUGAGGCCGAAAGGCCGAA	AGGAGAUGCCUA	UAGGCAUCUCCUA	UGGCAGGAAGAA
HH	9604	UUUAUUGAGGCU	CUGAUGAGGCCGAAAGGCCGAA	AAGCAGUGGGUU	AACCCACUGCUUA	AGCCUCAUAAAA
HH	9610	GCAAGCUUUUUU	CUGAUGAGGCCGAAAGGCCGAA	AGGCUUAAAGCAG	CUGCUUAGCCUC	AAUAAAGCUUUG
HH	9614	CAAGGCAAGCTU	CUGAUGAGGCCGAAAGGCCGAA	AUUGAGGCUUAA	UUAAAGCTUCAUA	AAGCUUGCCUUG
HH	9625	CUUGAAGCACUC	CUGAUGAGGCCGAAAGGCCGAA	AGGCAAGCUUUA	UAAAGCUUGCCUU	GAGUGCTUCAAG
HH	9639	ACGGGCACACAC	CUGAUGAGGCCGAAAGGCCGAA	ACUUGAAGCACU	AGUGCTUCAAGUA	GUGUGUGCCCGU
HH	9652	AGUCACACAACA	CUGAUGAGGCCGAAAGGCCGAA	ACGGGCACACAC	GUGUGGCCCGUC	UGUUGUGUGACU
HH	9665	CUCUAGUUACCA	CUGAUGAGGCCGAAAGGCCGAA	AGUCACACAACA	UGUUGUGUGACUC	UGGUAACTUAGAG
HH	9680	AAGGGUCUGAGG	CUGAUGAGGCCGAAAGGCCGAA	AUCUCUAGUUAC	GUAACTUAGAGAU	CCUCAGACCCUU
HH	9693	CCACACTUGACTUA	CUGAUGAGGCCGAAAGGCCGAA	AAGGGUCUGAGG	CCUCAGACCCUUU	UAGUCAGUGUGG

Table VIII:

nt. Position	Hairpin Ribozyme Sequence	Substrate Sequence
121	CACCAUCCAAAG AGAA GUGG ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	CCACU GAC CUUUGGAUGGUG
270	GAAUUGCUAGGC AGAA GUCA ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	UGACA GCC GCCUAGCAUUC
273	GAUGAAUUGCUA AGAA GCUUG ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	CAGCC GCC UAGCAUUUCAUC
515	UAUUGAGGCCUUA AGAA GUGG ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	CCACU GCU UAAGCCUCAUA
565	AGUCACACAACA AGAA GGCA ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	UGCCC GUC UGUUGUGUGACU
565	AGUCACACAACA AGAAGGCACA ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	UGCCC GUC UGUUGUGUGACU
2025	UUCACAUUUCC AGAA GCCC ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	GGGCU GUU GGAAUUGUGGAA
3273	GGCAGCACUAUA AGAA GUAC ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	GUACA GCC UAUAGUGCUGCC
3720	UCCUUUGUAUG AGAA GUUU ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	AAACU GCC CAUACAAAAGGA
4039	UGCAUUAUUGUA AGAA GUUA ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	UAACA GAC UCACAAUAUGCA
4398	GCCAUUAUCCUG AGAA GCAG ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	CUGUA GUC CAGGAAUAUGGC
4398	GCCAUUAUCCUG AGAAGCAGUC ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	CUGUA GUC CAGGAAUAUGGC
4612	CGCCACCAACA AGAA GCCC ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	GGGCC GCC UGUUGUGGGCG
5348	AUGAAUUAUUG AGAA GUUA ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	UAGCA GAC CAACUAAUUCAU
5379	CAGAGUCUGAAA AGAA GUCA ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	UGACU GUU UGUUCAGACUCUG
5760	UGAAUUGGAUA AGAA GCAG ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	CUGCU GUU UAUCCAUUUUCA
6334	UACCCCAUAUA AGAA GUGA ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	UCACA GUC UAUAUUGGGUA
6454	UUGUGGUGUGG AGAA GUGG ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	CCACA GAC CCCAACCCACAA
7013	CUUCUUCUGCUA AGAA GCCA ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	UGGCA GUC UAGCAGAAGAAG
7346	CUCCACAUAUA AGAA GUGC ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	GCACA GUU UUAUUGUGGAG
7392	CAAGUACUAUA AGAA GUUG ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	CAACU GUU UAAUAGUACUUG
7819	CAGCGUCAUUA AGAA GCGC ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	GGCA GCC UCAUUGACGCTUG
7833	CUGGCCUGUACC AGAA GCGU ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	ACGCU GAC GGUACAGGCCAG
7930	CUUGAUGCCCCA AGAA GUGA ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	UCACA GUC UGGGGCAUCAAG
9205	CACCAUCCAAAG AGAA GUGG ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	CCACU GAC CUUUGGAUGGUG
9354	GAAUUGCUAGGC AGAA GUCA ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	UGACA GCC GCCUAGCAUUC
9357	GAUGAAUUGCUA AGAA GCUUG ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	CAGCC GCC UAGCAUUUCAUC
9599	UAUUGAGGCCUA AGAA GUGG ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	CCACU GCU UAAGCCUCAUA
9649	AGUCACACAACA AGAA GGCA ACCAGAGAAACACACGUGUGGUAUACCUUGGUA	UGCCC GUC UGUUGUGUGACU



FIG. 15b.

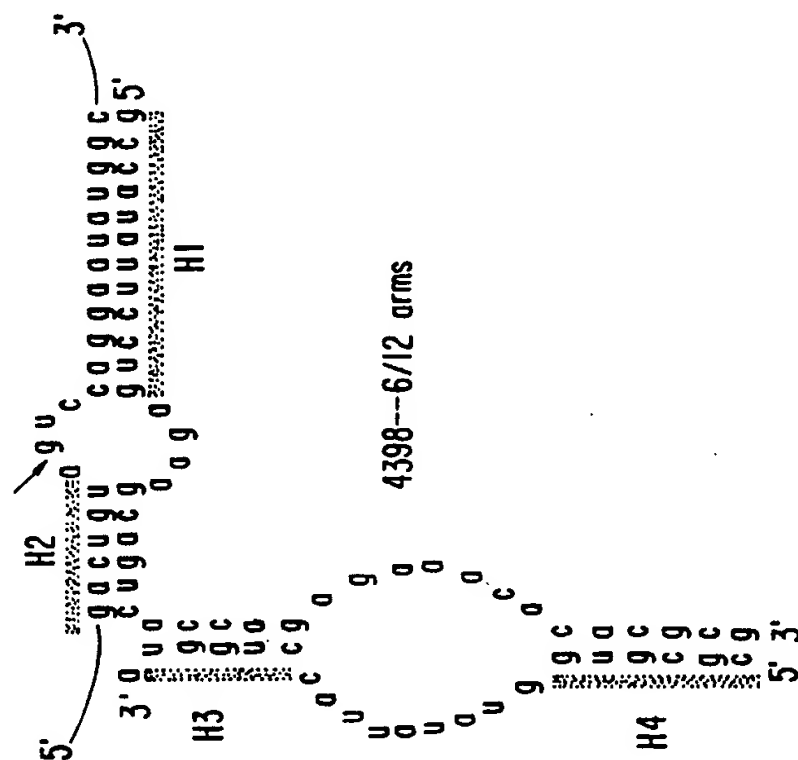
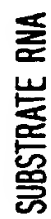
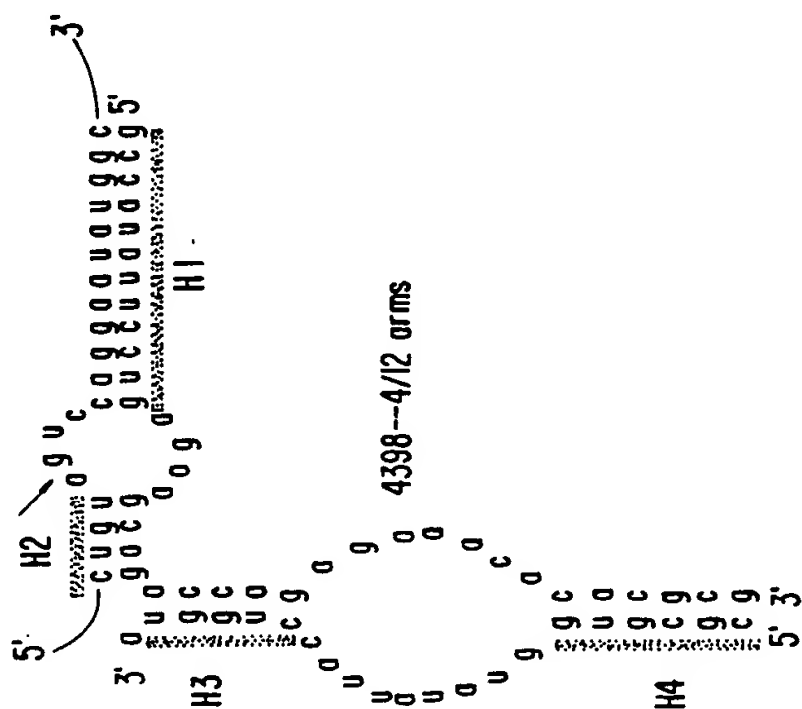


FIG. 15a.





## INTERNATIONAL SEARCH REPORT

Interns I Application No  
PCT/US 94/08613

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/11 C12N9/00 C12N7/04 A61K48/00 A61K31/70  
C12N5/10 C12N15/86

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,5 144 019 (ROSSI ET AL.) 1 September 1992 cited in the application see examples IV,V see Table 1, sequence number 11 ---	5-13,19
X	WO,A,93 05147 (THE SECRETARY DEPARTMENT OF HEALTH AND HUMAN SERVICES OF THE USA) 18 March 1993	14-18
Y	see page 8 - page 11, line 29 see page 45, line 1 - line 30 --- -/--	14-18

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

16 November 1994

Date of mailing of the international search report

01.12.94

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Andres, S

## INTERNATIONAL SEARCH REPORT

 Interns 1 Application No  
 PCT/US 94/08613

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF VIROLOGY, vol.66, no.3, March 1992 pages 1432 - 1441 DROPULIC, B. ET AL. 'Functional characterization of a U5 ribozyme: intracellular suppression of human immunodeficiency virus type 1 expression' cited in the application	20,21
Y	see figures 1,3,4 ---	14-18
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol.89, November 1992, WASHINGTON US pages 10802 - 10806 QJWANG, J. ET AL. 'Inhibition of human immunodeficiency virus type 1 expression by a hairpin ribozyme' cited in the application see figure 1 ---	20,21
X	ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, vol.616, 27 February 1991, NEW YORK, USA pages 184 - 200 ROSSI, J. ET AL. 'Ribozyme as therapies for AIDS' cited in the application see figures 6,7 see page 194 see page 198, paragraph 1 ---	20,21
A	---	1,3,8-10
X	NUCLEIC ACIDS RESEARCH, vol.20, no.17, 11 September 1992, ARLINGTON, VIRGINIA US pages 4581 - 4589 CHEN, C.-J. ET AL. 'Multitarget-ribozyme directed to cleave at up to nine highly conserved HIV-1 env RNA regions inhibits HIV-1 replication -- potential effectiveness against most presently sequenced HIV-1 isolates' see page 4586, right column, last paragraph - page 4588, left column, line 15; figure 2 ---	20,21
A	---	14-18
X	EP,A,0 360 257 (THE BOARD OF REGENTS OF THE NORTHERN ILLINOIS UNIVERSITY ET AL.) 28 March 1990 cited in the application see figure 15; example 19 see example 20 ---	20,21
2	---	
1	X WO,A,91 03162 (CITY OF HOPE) 21 March 1991 cited in the application see figure 1 ---	20,21
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## INTERNATIONAL SEARCH REPORT

Intern al Application No  
PCT/US 94/08613

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,91 04319 (INNOVIR LABORATORIES, INC.) 4 April 1991 cited in the application see examples 4-6 ---	8-11, 14-18
A	ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, vol.660, 1992, NEW YORK, USA pages 95 - 106 ZAIA, J. ET AL. 'Status of ribozyme and antisense-based developmental approaches for anti-HIV-1 therapy' cited in the application ---	
P,X	WO,A,93 23569 (RIBOZYME PHARMACEUTICALS, INC.) 25 November 1993 cited in the application see examples 2,15 see figure 7 see claims ---	1-21
P,X	NUCLEIC ACIDS RESEARCH, vol.21, no.22, 11 November 1993, ARLINGTON, VIRGINIA US pages 5251 - 5255 CRISELL, P. ET AL. 'Inhibition of HIV-1 replication by ribozymes that show poor activity in vitro' see figure 1 -----	5

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 94/08613

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 12-13, 19 and 14-16(as far as in vivo methods are concerned) are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

information on patent family members

Intern al Application No

PCT/US 94/08613

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5144019	01-09-92	US-A- 5272262	21-12-93
WO-A-9305147	18-03-93	AU-A- 2512792	05-04-93
EP-A-0360257	28-03-90	AU-B- 641900	07-10-93
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		AU-A- 5817790	08-04-91
		DE-T- 4091533	30-01-92
		EP-A- 0451221	16-10-91
		EP-A- 0506666	07-10-92
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		JP-T- 5502999	27-05-93
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		AU-B- 4221593	13-12-93